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Biology and Physiology of the Lower Trypanosomatidae

R. BARCLAY McGHEE* AND WILLIAM B. COSGROVE

Department of Zoology, University of Georgia, Athens, Georgia 30602

INTRODUCTION

The family Trypanosomatidae of the phylum Protozoa is characterized by a single axoneme and by an organelle known as the kinetoplast, a mitochondrion rich in deoxyribonucleic acid (DNA). Of the nine recognized genera, two are known to include species pathogenic for humans and animals (*Leishmania* and *Trypanosoma*), and some species (*Phytomonas*) are recognized as pathogens of plants.

The first record of a member of the family was that of Leidy (164), who assigned a specific name (*Bodo muscarum*) to an organism discovered in the house fly, *Musca domestica* Linnaeus. In 1880 Kent (138) established two genera, *Herpetomonas* and *Leptomonas*, on the basis of past records in the literature. Included in the former genus was an organism observed in the blood of a rat, which is now known to be a member of the genus *Trypanosoma*. The general similarities among the various genera, as exemplified by Kent, resulted in many misconceptions and ultimately in a great deal of confusion as to the taxonomy of the group and a lack of understanding of the pathogenic proclivities of the family members.

The review by Wenyon (286) in 1926 was an excellent critical analysis of the state of knowledge of the family. The review by Wallace in 1966 (281) included a carefully worked out revision of the group but did not include a consideration of the genus *Phytomonas*. Since that review, there has been a renewed interest in the lower Trypanosomatidae stimulated not only by the review of Wallace, but also by the belief that these organisms might serve as models for the study of pathogens of vertebrates and be suitable for testing effective chemotherapeutic agents (7, 94, 139). The pathogenic propensities of *Phytomonas leptovasorum* Stahel toward *Coffea* sp. (253) and of the recently discovered *Phytomonas staheli* McGhee and McGhee (180), which is wreaking havoc on oil palms (*Elaeis guineensis*) and coconut palms (*Cocos nucifera*) (61, 205), demand that more attention be placed on these serious diseases of plants of great economic importance. The possible zoonotic proclivity of this group of flagellated protozoa, which was first suggested by Laveran and Franchini (160) and exemplified recently by a possible infection of a human (McGhee, unpublished data), along with the recent discoveries that other protozoa that are free-living or parasitic in lower animals can parasitize humans (33, 108), has prompted a reevaluation of the role of the lower trypanosomatids as putative parasites of vertebrates. Although some doubt has been raised recently about the adequacy of some species as models (90), their increasing use in bioassays (22, 78, 225), as sources of enzymes for biochemical and clinical research (40, 139), and in the diagnosis of certain diseases (49, 52, 283) emphasizes the importance of these organisms. Moreover, any real understanding of trypanosomatids in general demands an understanding of the less specialized species as thorough as our understanding of the more specialized *Leishmania* and *Trypanosoma*. We present here a review of the current (August 1979) state of knowledge of lower trypanosomatids in the hope that the usefulness of these organisms as experimental material will be brought to the attention of a much-enlarged group of biologists.

In this review we have omitted, because of a lack of space, considerations of cultivation, nutrition, and energy metabolism, subjects for which recent reviews or summary accounts exist (73, 97, 172, 197, 268, 271). We have also not considered the related free-living bodonine flagellates, for which the reports of Brooker (15) and Vickerman (279) can be consulted. Vickerman and Preston (280) also provide an excellent general account of the order Kinetoplastorida, to which all of these flagellates belong.

GENERAL BIOLOGY AND SYSTEMATICS

Systematics

The classification of these organisms is as follows: phylum, Protozoa; subphylum, Sarcomastigophora; superclass, Mastigophorasica; class, Zoomastigophorasida; order, Kinetoplastorida; family, Trypanosomatidae.

In the phylum Protozoa, there is uncertainty as to the definition of the higher taxa. This uncertainty also extends to the generic and species levels. The family Trypanosomatidae has had its share of taxonomic difficulties, engendered by perhaps unavoidable mistakes, including misinterpretation of morphological stages, mixed infections, and uncontrolled experimentation.

Fortunately, the genera most directly affecting humans, *Leishmania* and *Trypanosoma*, have been studied intensively; there is little confusion about generic status, although species determinations in the *Trypanosoma* "*brucei*" group of African trypanosomes, as well as criteria for naming species of *Leishmania*, continue to be a problem for investigators (120, 238).

Although the shapes of members of the family may vary from oval to pyriform and the lengths may vary from 4 to 385 μm, the suborders Trypanosomina and Cryptobiana share a structure known as the kinetoplast. For years this was an enigmatic structure; however, the development of electron microscopy allowed its visualization as a portion of a single mitochondrion rich in DNA.

A single flagellum is a nearly constant feature. The basal body of the axoneme is close to the kinetoplast but is not attached to it. In certain herpetomonads of *Drosophila* sp. an anomalous condition exists. In some the axoneme terminates in an area approximating that of a typical promastigote. The kinetoplast is, however, situated posteriad to the nucleus. In other herpetomonads the kinetoplast is in the area generally occupied in the promastigote, but it is bypassed by the axoneme which terminates in the posterior end of the body. The relative positions of the kinetoplast and the axoneme constitute important diagnostic features of the various genera (37). Until the revision of morphological forms by Hoare and Wallace (121) (Fig. 1), the stages were known by their generic appellations, i.e. leptomonad, leishmanial, etc. Since the work of these authors appeared, two or perhaps three additional forms have been described. One, the paramastigote, represents in all probability a transitional stage between a promastigote and an ophisthomastigote. The spheromastigote is seen in certain populations of flagellates, whereas the endomastigote of Yoshida et al. (291) and the opisthomastigoid of Riley (229) are quite similar to a form described by Wenyon as a cystic stage of *Herpetomonas* (286).

A combination of forms and the respective plant and animal hosts provides us with a definition of genera (Fig. 2).

Although the criteria for establishing genera are more or less straightforward, the problems of designating species remain. Structural differences are not sufficient to be of any real assistance. Caution must be exercised in establishing species on the basis of host specificity of trypanosomatids. It is generally accepted that the Trypanosomatidae are asexual. Thus, the criterion of sexual isolation is of no use as an aid to species determination.

Early efforts to designate species were limited to the study of Noguchi and Tilden (198), who utilized fermentative properties and serological characterizations to describe four new species of *Herpetomonas*. The successful cultivation of numbers of species of the Trypanosomatidae (181, 197) and especially the monophasic cultivation of the genus *Crithidia* (47) have permitted more exact methods of differentiation of species. Beginning in 1959 (174), McGhee and colleagues made an intensive study of the characteristics of members of the family with a view

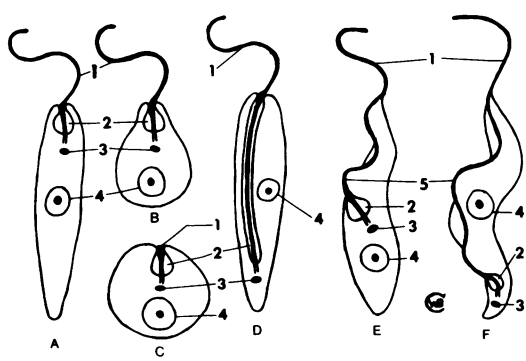

FIG. 1. *Stages of Trypanosomatidae. A, Promastigote; B, choanomastigote; C, amastigote; D, epimastigote; E, opisthomastigote; F, trypomastigote; 1, flagellum; 2, flagellar pocket; 3, kinetoplast; 4, nucleus; 5, undulating membrane.*

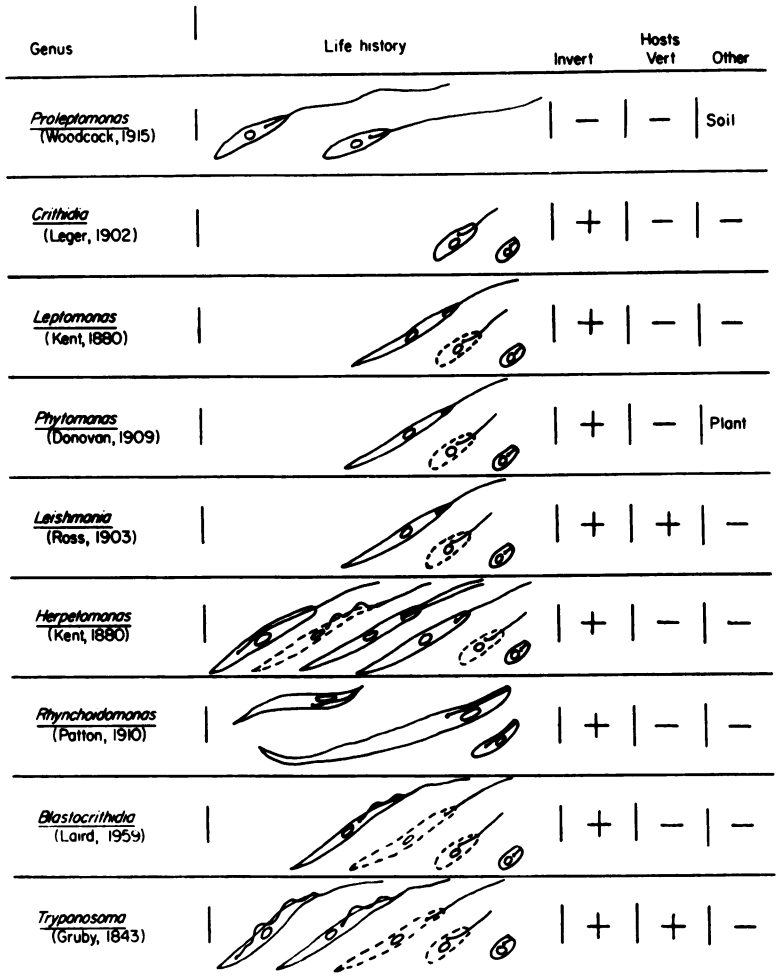

FIG. 2. *Schematic diagram of genera of Trypanosomatidae. The figures depicted by broken lines represent stages reported but not confirmed. Reproduced from reference 174a.*

toward separation of species. Ultimately, 10 cloned isolates of *Crithidia* were studied in relation to (i) host specificity, (ii) serology, and (iii) growth in acidic and basic media (178). Two or probably three species were from Diptera, six were from Hemiptera, and one was from Hymentoptera.

Serology (agglutination) itself divided the isolates into groups reflecting the order of the host (i.e., Hemiptera, Hymenoptera, or Diptera). Previous studies (103, 104, 236) involving host specificity, although emphasizing the loose host specificity of the genus, did indicate differences in growth in avian embryos and in five insect species. Growth of the 10 isolates in acid and basic media provided a final separation of the species.

It may have been entirely fortuitous that the 10 species selected for the study responded so clearly to the series of differential tests. It is possible that newer representatives subjected to the same battery of tests might not respond so precisely. In this study the organisms were cloned, and it is possible that more than one species of *Crithidia* were represented in the population from which a clone was obtained. Ironically, the motive for searching for more adequate methods of species differentiation (i.e., lack of host specificity) failed to be a factor. As others had thought, each *Crithidia* from each different host constituted a species. This discovery led to a revised concept of host specificity (see below).

Dooris and McGhee (62) introduced a refinement of immunological and electrophoretic techniques for determining species differences. When *Crithidia fasciculata* from Diptera and *Cri-*

thidia harmosa from Hemiptera were compared by using indirect immunofluorescence and agglutination, major differences were detected between the two organisms. Electrophoretic patterns obtained by polyacrylamide gel slab electrophoresis indicated similar differences.

Although this represents a promising beginning, it is still far from being a solution to the problem of species separation in the Trypanosomatidae. Few other genera are capable of growth and multiplication in monophasic media. Many species defy cloning or even cultivation. Therefore, anyone interested in the taxonomy of the group should continue to utilize such characteristics as hosts, life cycles, population cycles, and morphology as aids in establishing species. If these methods are complemented by the powerful methods of molecular biology (DNA-DNA hybridization, electrophoretic analysis of isozymic patterns, detailed comparisons of specific proteins [1, 115, 117, 118, 153, 154, 169, 216–218, 260]), many of the vexing problems of recognition and definition of species could be solved. For example, Hill and Pettigrew (118) have shown that the closely related species *C. fasciculata* and *Crithidia oncopelti* have cytochromes *c* differing in 13 amino acid positions; Steinert et al. (260) have shown that autoradiographic methods can be used to differentiate between kinetoplast DNAs of different species in cytological preparations. This technique is applicable even to those putative species which have not been grown in culture.

Examination of Genera

The genus *Proleptomonas* was first described by Woodcock in 1916 (290) from a culture of goat feces. The organism is depicted with a kinetoplast and may be merely a free-swimming form of *Leptomonas* or *Herpetomonas*. A total of 58 species of *Leptomonas* Kent have been described since the discovery of this genus in a free-living nematode in 1919. The genus *Crithidia* Léger was first observed in the gut of an anopheline mosquito. Associated with this organism was the genus *Blastocrithidia* Laird. A total of 15 species of *Crithidia* are now listed, and there are 30 species of *Blastocrithidia* recognized.

Rhynchoidomonas Patton may be merely a variant of the genus *Herpetomonas* Kent. Only 5 species of *Rhynchoidomonas* are known, but 30 species of *Herpetomonas* are recognized.

The genus *Phytomonas* Donovan was discovered by Lafont in 1909. There are probably six species in the genus, each from a different family of plants. It is a dioxenous genus; the known insect vectors are all hemipterons (177, 222). Recently, *Phytomonas davidi* of Euphorbiaceae was cultured in a diphasic medium containing duck blood and Cowperthwaite medium (47).

Distribution

It is readily apparent that, with the exception of four species of *Leptomonas*, all 123 monoxenous members of the Trypanosomatidae have as hosts members of the phylum Arthropoda (Table 1). One species of *Blastocrithidia* occurs in ticks (199). Most species are found in Insecta. Diptera and Hemiptera serve as hosts for 102 species. The orders Hymenoptera, Lepidoptera, Orthoptera, and Siphonaptera contain the hosts of the remaining 21 representatives. One species of *Leptomonas* is found in a marine nematode (34), and still another is found in the macronuclei of *Paramecium* (85). This known distribution may simply represent too few examinations of other groups of potential hosts or a concentration on those insects containing vectors of human diseases.

All orders of insects serving as hosts are thought to have arisen during the Pennsylvanian period and are among the most highly evolved groups. Ixodidae is parasitized by three species of *Blastocrithidia*. Individual Diptera and Hemiptera may be infected with three or even more genera. In honey bees (*Apis mellifera*) (156) and in yellow jackets (*Vespula squamosa*), only *Crithidia* species have been found.

Somewhat the same situation exists for the phytomonads. Again, there is the possibility that inadequate sampling of plants has led to the discovery of *Phytomonas* in only four orders of angiosperms. Most records are of infections of laticiferous plants. Infections of palms and coffee were discovered as results of the diseased conditions of the host plants. Other reports of parasitism of the Solanaceae (82) have resulted from examinations of puncture wounds of fruits.

The orders Principes in the monocotyledons

TABLE 1. *Distribution of Trypanosomatidae of arthropods relative to host organism*

Arthropod taxon	No. of species				
	Leptomonas	*Blastocrithidia*	*Crithidia*	*Rhynchoidomonas*	*Herpetomonas*
Diptera	23	3	10	5	10
Hemiptera	24	18	8	0	1
Siphonaptera	5	3	0	0	0
Ixodidae	0	3	0	0	0
Hymenoptera	0	1	3	0	1
Lepidoptera	1	0	1	0	1
Orthoptera	2	0	0	0	0
Others[a]	3	0	0	0	0

[a] Including Nematoda, Anoplura, and Homoptera, in which less than two species have been described or where doubtful records exist.

and Rubiales, Gentiniales, and Euphorbiales in the dicotyledons are considered to be among the most highly evolved orders of plants.

The few known invertebrate hosts of *Phytomonas* are all hemipterons; most belong to the family Lygaeidae.

Many successful attempts to induce infections in other insects by either feeding (81), intrahemocoelic injection, or intrarectal injection have been reported (284). There have been few efforts to infect plants with *Phytomonas*. Members of the Euphorbiaceae were refractory to infection with *Phytomonas elmassiani* (106), whereas two species of Apocynaceae, *Allamanda nereifolia* and *Plumeria rubra*, were susceptible. Infections in these presumably foreign hosts were marked by the presence of choanomastigotes, reflecting, perhaps, a profound influence of the exotic hosts on the morphology of the parasites.

Ecology

Little if any attention has been given in the past to the epizootiology, host specificities, or even life cycles of the lower trypanosomatids. The concept of transmission from host to host seems to have been that such transmission is a matter of chance contamination of a putative host. Although it was generally believed that there was some host specificity involved, few efforts to verify such beliefs were made. Relevant studies (81, 283) suggested that there was a rather loose host specificity. A series of investigations beginning in 1963 clarified these issues somewhat and resulted in a new definition of host specificity and a concept of parasite survival through contiguity of host and parasite life cycles.

Life histories. A characteristic shared by the hosts of the trypanosomatids is piercing mouth parts. Exceptions to this are Mecoptera (scorpion flies), Trichoptera (caddisflies), Orthoptera (roaches), and, in part, Hymenoptera. Most orders possessing piercing mouth parts contain representatives which are hosts of the Trypanosomatidae. There are two notable exceptions, Thysanoptera (thrips) and Homoptera (plant lice).

Until recently, transmission of trypanosomatids of arthropods was thought to occur by fecal contamination. Ovarian transmission was postulated by Porter (221), O'Farrel (199), Rodhain et al. (230), and Lipa et al. (168). Usual transfer has been presented as an indiscriminate dispersal of the trypanosomatids in a fecal vehicle. Infection of originally parasite-free hosts would thus be relatively rare, too rare indeed to allow a population infection rate of 40%, as seen in feral *Drosophila melanogaster* during certain times of the summer (41). The infection rate of *Oncopeltus fasicatus* with *Leptomonas oncopelti*, although lower, reflects a precise rather than a random method of passage of parasites from individual to individual. There are additional problems when arthropods with sucking mouth parts are involved. Such a host must chance upon a contaminated twig or leaf and somehow ingest the parasite in the process of inserting its beak through the plant epidermis.

If a given host were susceptible to all species of Trypanosomatidae, the possibility of insuring the existence of the parasitic species would be enhanced. Such loose host specificity appears to exist in the genus *Crithidia* (104). Thus, *O. fasciatus* underwent infections when species of *Crithidia* were introduced by either ingestion or intrarectal injection. Similar findings were reported by Garnham (81) and Wallace and Johnson (283) when various species of mosquitoes were allowed to feed on material containing *C. fasciculata*. An even wider array of hosts (Orthoptera, Coleoptera, Diptera, and avian embryos) became infected after receiving inocula of each of six species of *Crithidia* (236).

When *O. fasciatus* was infected intrarectally with *Trypanosoma cruzi*, the etiological agent of Chagas' disease, the parasite flourished (104). Another parasite of humans, *Trypanosoma rangeli*, multiplied well when it was injected into the hemocoel of the same bug. It was also determined that when *Blastocrithidia euschisti*, a monoxenous trypanosomatid from a hemipteron (*Euschistus servus*), was injected intrarectally into *Triatoma infestans* and *Rhodnius prolixus*, the parasite survived and multiplied. These findings prompted a warning to those working with *T. cruzi* and the Triatominae of potential infections of laboratory bugs with members of the lower Trypanosomatidae. Shortly thereafter, laboratory bugs were indeed found to be infected; the parasites were transmitted through the bugs in the colony (26, 27, 54).

If such a loose host specificity exists it is difficult to reconcile a one-to-one relationship between *Crithidia* and host. Experiments (178) demonstrated that 10 isolates of *Crithidia* were indeed different species. During the process of studying these characteristics, the question was posed as to whether the serological grouping of a parasite (i.e., dipteron, hemipteron, or hymenopteron) might be changed by a prolonged sojourn of the parasite in a host having a serology different than its own serology. An experiment was carried out in which *C. fasciculata* was transmitted intrarectally through several generations of *O. fasciatus*. After a 1-year existence in the hemipteran host, the serological typing of the parasite remained affiliated with its dipteran antecedents.

None of the offspring of experimentally infected *Oncopeltus* developed infections. In only a single instance, one in which *O. fasciatus* was injected with a blastocrithidian from a reduviid hemipteron (*Melanolestus picipes*), was there transmission to nymphal *Oncopeltus*. It appeared, therefore, that infections of dipteran origin were accidental and that transfers to offspring would be unsuccessful.

To test this hypothesis, *D. melanogaster* was confined in breeding jars with *Drosophila robusta*. The latter species was parasitized with a naturally occurring *Crithidia*. After 1 week, a sample of the hitherto trypanosomatid-free, but not infected, *D. melanogaster* was removed from contact with *D. robusta* and placed in a population of claret-eyed mutant *D. melanogaster*. Although other claret-eyed mutants growing with infected *D. robusta* became infected, those mixed with the infected wild-type *D. melanogaster* remained parasite-free.

In both experiments, the *Crithidia*, although readily parasitizing the various hosts, were not transmitted from parent to offspring. It is apparent, therefore, that there is a rather strict host specificity, at least as regards *Crithidia*. Moreover, it suggests that the definition of host specificity should be altered from susceptibility of a given host to establishment of a parasite (i.e., transmissibility from parent to offspring).

If the concept of narrow host specificity of the Trypanosomatidae is accepted, one must at the same time postulate some more logical manner of transmission from host to host. To formulate such a hypothesis, the life cycles of a group should be examined with a view toward discovery of a method or methods of transmission.

Crithidia sp. of *D. robusta*, as is true of all species of *Crithidia* studied, exists in the choanomastigote stage and what might be called a semiamastigote stage. In addition to apparent resistant stages of *C. oncopelti* (4), free-swimming stages of *C. fasciculata* have been observed in water occupied by its host, the mosquito *Culiseta incidens* (37).

In one experiment (McGhee, unpublished data) *D. robusta* adults were allowed to feed on cotton soaked with cultured *Crithidia* isolated from this species of fly. After 2 days one *D. robusta* was dissected and found to be infected; 3 days later the banana mash serving as food for the caged flies was examined, and free-swimming choanomastigotes were observed. The experiment was repeated, and swimming flagellates were again detected in the banana mash.

Under these conditions and under those used by Clark et al. (38), free-swimming *C. fasciculata* cells are found in the same habitat and at the same time as the host, as either adults, larvae, pupae, or eggs. Therefore, there is ample opportunity for continuous exposure of host to parasite; in other words, a contiguity of life cycles of host and parasite exists. Of course, this is only a simulation of natural conditions, but comparable conditions could occur in nature. The external habitats of the hosts of *Crithidia* parasites of the Culicidae genera *Gerris* and *Tabanus* are aqueous at some stage of host development, whereas the habitats of Muscidae and Drosophiliae are liquid or semiliquid. Infections of terrestial Hemiptera pose a different problem, one that may be partially clarified by an examination of factors affecting transmission of *Leptomonas*.

Of the 55 species of *Leptomonas*, the life cycles of only 7 have been elucidated even partially. One, *Leptomonas jaculum*, in the aquatic hemipteron *Nepa* sp. is reported to infect the ovaries of its host (221) and, as was reported to be the case for *Blastocrithidia hyalomae* (199), is assured of survival into offspring. *Leptomonas chattoni* of the moth *Agrotis* sp. (202) is found in the hemocoel of its host. It may well be that hemocoelic invasion may be more common than supposed. *L. oncopelti* has been observed in the hemocoel of *O. fasciatus*. *Leptomonas serpens* (83) is reported to infect tomato fruits as well as its insect host; the pentatomid hemipteron *Nezara* sp. Wenyon (286) succeeded in transmitting *Leptomonas pedicula* of the human louse *Pediculis humanis* via feces of infected hosts. In our laboratory (103) *Leptomonas leptoglossi* was transmitted from parent bug to offspring. The early developmental stages of the leptomonad in the nymphal bugs were quite enlarged, reminiscent of similar forms recorded in infections of *O. fasciatus* with this organism.

A more detailed study of the life cycle of *Leptomonas ctenocephali* was made by Wenyon (286). This parasite exists as attached and free promastigotes and amastigotes in the alimentary tracts of dog fleas (*Ctenocephalus canis*). Amastigotes are resistant to external environments and are deposited via feces in the litter where the fleas oviposit. The larval fleas feed to a great extent on the nutritive feces of the adults. The excysted leptomonads are found in developing larvae and survive the development of the hosts to adulthood.

L. oncopelti undergoes a life cycle marked by unequal cytokinesis as well as binary fission (176). In laboratory-cultured bugs, *Leptomonas* is found within 2 weeks of hatching. Although infections are quite heavy, no flagellates appear in the feces of these bugs, a result of incomplete patency of the posterior portions of the digestive tracts of the nymphs. In the anterior intestine and pylorus, divided nuclei separate, and one or

more move to the anterior portion of an organism. Apparently, the position of the nucleus influences cytokinesis; these organisms divide by budding. Quite often the resultant small organisms ("strap hangers") remain attached to a parent flagellum. Additional divisions (this time equal) may ensue. The moment the adult bugs appear, the alimentary tracts become patent, and the parasites descend to the recta, where apparently only the smaller amastigotes survive.

Feces deposited by infected adults are usually well supplied with amastigotes; these feces retain their infectiousness after drying at 25, 37, and 45°C for prolonged periods of time, although no survival was observed after exposure to 55°C. In the laboratory, infections are transmitted from bug to bug through fecal contamination of the water source, food source, or fomites.

Nymphs emerging from eggs of infected adults became infected even though the egg masses were removed from the other adult bugs immediately after ovipositing. In one instance an infected female bug thrust her ovipositor through netting covering the jar and deposited eggs on the outer side. The nymphs which emerged from this egg mass eventually became infected. If egg masses from infected bugs were separated and allowed to dry, nymphs failed to become infected. Inasmuch as treatment of egg masses with White solution resulted in the absence of infection in hatched nymphs, the possibility of ovarian transmission was thought to be unlikely.

Egg masses are held together by a material which retains it glutinous character until hatching. Simultaneous hatching is rarely realized. First-born nymphs cannabalize unhatched eggs. A bug moves over the egg mass, salivating as it goes. Eventually a victim is selected, the proboscis is inserted, and the shrunken abdomen of the nymph becomes distended. The bug does not begin to eat seeds and drink water for 24 to 48 h. It is not known whether the exteriors of eggs are contaminated with feces or whether parasites are deposited in some other manner. However, in one instance *Lygaeus kalmii*, another lygaeid hemipteron infected with its own *Leptomonas*, covered its egg mass with debris cemented together by feces.

In nature the possibility of indiscriminate fecal contamination being responsible for transmission is more remote. Infection of nymphs as a result of cannibalism of contaminated eggs is more likely.

Whether this method of transmission occurs in other species of Trypanosomatidae is unknown. It is known, however, that certain Hemiptera (*Leptoglossus phyllopus*, *Arilus cristatus*, *Acanthocephala femorata*) which harbor trypanosomatids do lay eggs in a mass. The habits of others relative to egg laying are as yet not known.

Again, there is a condition wherein eggs, nymphs, and adults of a host insect are in constant contact with the infecting protozoan. Survival of *L. oncopelti* is assured by contiguity of the life cycles of host and parasite.

Our knowledge of the life cycles of the 30 recognized species of the genus *Blastocrithidia* (281) is not complete. O'Farrel (199) described the occurrence of multiple fission and the presence of amastigotes in the ovaries of infected *Hyalomma* and postulated ovarian transmission to young ticks. Gibbs (82) described budding and formation of flagellar cysts in *Blastocrithidia familiaris*.

Although complete life cycles of *Herpetomonas* have yet to be elucidated, the work of Wallace (282) represents a beginning that should eventually lead to a full knowledge of these organisms. His studies stressed the existence in this genus of three distinct forms, opisthomastigotes and uni- and biflagellate promastigotes, and suggested that these forms represent parts of its life cycle. In some strains of *Herpetomonas muscarum*, opisthomastigotes occur in culture; in others they are rare. Subjecting opisthomastigote-rich cultures to refrigeration, to urea, and to higher temperatures increases the percentages of these forms. In addition, loss of infectivity of *H. muscarum* from cultures was correlated with similar phenomena in *Trypanosoma*.

New light was shed on the life cycle of *Herpetomonas* by Fleming (74), who attempted to explain the virtual absence of *H. muscarum* in *Musca domestica* during the winter months. The high incidence of infection in the warmer months and its absence when the mean temperature was low suggested the possibility of a temporary existence of some form of the flagellate in the external environment. The concept was reinforced when it was found that transmission within the laboratory was greatly enhanced when artificial garbage was added to the cages holding *Musca*. Brun (22) found that *H. muscarum* flagellates could survive for 16 h in meat on which they were deposited in feces. There was no mention of free-swimming stages in this environment. Brun was convinced that the development of *H. muscarum* in the flesh-eating fly *Chrysomyia* sp. is complex and includes as a portion of its life cycle the organism hitherto known as *Herpetomonas mirabilis*. Whether there are two species or merely one has not yet been determined. The postulated life cycle is, however, reminiscent of that of *Herpetomonas ampelophilae* (see below).

Becker showed that transmission from fly to fly is possible (11); he observed infections in

house flies after they fed on carcasses of flies infected with *Herpetomonas*. Confirmation of the existence of free-living stages was obtained by Riley (229), who found motile flagellates in the juices of an apple on which feral *M. domestica* was feeding. When cultured herpetomonads were placed in a standard house fly larval medium, they survived for 36 h. Forms resistant to drying occur in *Crithidia* (4).

Corwin (41) observed herpetomonads swimming freely in the banana mash used as a medium for developing larvae of *D. melanogaster*. Rowton and McGhee (235) maintained an agelededeme (i.e., a population of parasites in a population of hosts) of *H. ampelophilae* by maintaining the flies in population cages provided with a constant supply of fresh medium. Riley (229) detected living *H. ampelophilae* in milk, yeast, potato, and grape solutions from 12 to 24 h. It is possible that an organism described as *Proleptomonas terricola* is actually only a free-swimming *Herpetomonas* (290).

H. ampelophilae undegoes a rather complex cycle within a host. Parasites may be present only in the endotrophic spaces, in peritrophic areas, or in the Malphigian tubules. Although transmission in the laboratory may be by contamination of the food or water supply (as obtained in transmission of *L. oncopelti* in laboratory-reared hosts), it may not represent natural conditions. In nature, concentrations of hosts comparable to those present in laboratory cultures of *Musca* or *Drosophila* are rarely, if ever, obtained. Therefore, although infections of *Drosophila* reared in the laboratory may reach 100% and rarely fall below 90%, infections of feral *Drosophila* rarely reach 40%. There is the possibility that in nature and in the absence of concentrated populations of hosts, an abundance of food sources, and free-swimming parasites, some other method of transmission occurs.

The possibility of such a life cycle is supported by the demonstration of a transstadial infection of *H. muscarum* (229). The survival of organisms through the pupal stage, when virtually all of the internal organs are dissolved, had been questioned. This study, however, reported living flagellates in the "floating yellow body" (214) or meconium of pupae. This body persisted until eclosion of the imago. Parasites were then found in the Malphigian tubules and eventually in the guts of adult flies. They were also observed in the meconia of *Megaselia scalaris* and *Phormia regina*, as well as in *D. melanogaster*.

No true cysts have been described for *Herpetomonas*, although Wenyon (286) interpreted as a cyst a modified opisthomastigote which had no external flagellum. A similar form, which possessed a minute flagellum and was called an opisthomastigoid, was observed in the meconia of house flies. Yoshida et al. (291) observed the form observed by Wenyon in populations of *Herpetomonas mariadeani* and called it an endomastigote. It is reminiscent of the "stumpy" trypomastigotes of *Trypanosoma*. It may be a "transmission stage" of *Herpetomonas*.

Thus, in *Herpetomonas* as in *Leptomonas*, transmission is a precise rather than a random process.

The foregoing accounts of life cycles, although admittedly covering only a small section of the Trypanosomatidae, indicate precise methods of transmission, methods which provide the parasites with assurances of furthering the species. Involved in all cycles studied so far is the continual provision of a liquid phase during some stage of the development of the parasites. Perhaps the simplest manifestation of the life cycle is the life cycle in which a host organism exists in an aquatic habitat. Thus, species of *Crithidia* found in Culicidae are shed from the confines of the mosquito larvae and undergo a free-living stage in water (37). A similar cycle might be postulated for *L. jaculum* in the water scorpion *Ranatra* sp. (221).

If an arthropod (or plant) is removed from its aquatic environment, other more sophisticated methods of transfer become necessary. Again, the intestinal tract of a host provides a fluid phase for that stage of development. At some developmental stage of the host the external environment is liquid. Thus, *H. ampelophilae* of *Drosophila*, *H. megaseliae* of *Megaselia* sp., and *H. muscarum* of *Musca* exist in free-living states in the larval media, which coincidentally are sources of food for the adults as well as the larvae. In the above examples transmission reflects some dependence on the gregariousness of the host.

Crithidia sp. of *D. robusta* utilizes the medium of the larvae to spread its free-living stages in much the same way as *C. fasciculata* swims in its aqueous environment. The method of transmission of *Crithidia mellificae* (156) of honey bees is not known, although it may pass from adults to larvae through the liquid medium provided. An additional means of transfer might be through liquid food exchange (trophallaxis) among members of the same colony. This involves not only exchange of food from adult to adult but a reward excretion by larvae in response to feeding by an adult *Vespula* sp. (288). In these examples the parameters of transmission may extend to the behavior of the hosts. Although a *Crithidia* has been isolated from this latter host, no details of its life history are available (R. B. McGhee and S. Urdaneta, unpublished data).

However, when there is no available liquid medium for external survival, still another means of transmission is found. As described above, *L. oncopelti* spends its time outside its arthropod host in the glutinous material binding the egg mass together. Moreover, the parasite depends on cannibalism of the newly hatched nymphs for its continued survival. Transmission of *Blastocrithidia*, *Crithidia*, and *Leptomonas* of other terrestial Hemiptera may also utilize this route of transfer.

Utilization of egg masses for transmission of trypanosomatids of terrestrial arthropods offers a possible explanation of the virtual absence of trypanosomatids in the Homoptera. In this order of insects, oviposition is accomplished usually by insertion of a single egg rather than deposition of masses of eggs, as in Hemiptera.

Finally, a parasite may have no external environment. Thus, *Phytomonas* exists in two closed systems, insects and plants (6, 76, 125, 175). *Trypanosoma* and *Leishmania* live in comparable systems, but in two animal taxa. Perhaps the ultimate in assurance of transmission exists in *Trypanosoma equiperdum*, where transmission is venereal. There is no complete assurance of survival, however, since continued existence of this species of parasite is dependent on the caprices of equine sexual desire.

It is apparent that *P. elmassiani* and *Phytomonas davidi* do not undergo the hazards of an external existence. The completely obligatory nature of the dual association of the parasites with both plants and insects, however, does require nearly constant association. The limited range of *P. davidi* seems to be a function of the continued growth of members of the Euphorbiaceae and the lack of diapause in *Pachybrachius* (222). Only in the northern parts of their ranges do *Chamyaesyce hirta* and *Chamyaesyce hyssopifolia* fail to survive the winter. Consequently, there may be a limited migration of the insect vector, spreading the parasite to areas of plant die-back, or there may be reinfection of vernal plants by hibernating bugs. In areas where members of the Asclepiadaceae do not die back, as in the more tropical latitudes, the continued existence of a protozoan in an asclepiad might be expected. In areas north of the 24th parallel in the United States, all host plants die back to their roots (123). Absence of latex tubules in the root stocks, annual regrowth from crowns other than the ones of the previous year, and winter absence of the hemipteran vector produce environmental conditions even more severe for the survival of the parasitic species than do those acting against monoxenous species.

The hazard is overcome to some extent by an annual migration of the infected insect vector, *O. fasciatus* (179). Flights in the eastern part of the United States begin from the southern end of the Florida peninsula, where isolated ruderals of an exotic asclepiad survive the winter and harbor *Phytomonas*. Often, by late autumn bugs have moved as far north as the 42nd parallel, spreading infection to the various native Asclepiadaceae along the way. The first freeze disposes of parasites, bugs, and plants.

Common in all cases is the close association between the life cycles of host(s) and parasites. This phenomenon may or may not be true of the many species of invertebrate-infecting Trypanosomatidae, but its occurrence is nevertheless so consistent in the known life cycles that a principle of parasite survival through contiguity of life cycles of parasites and hosts can be postulated.

Pathogenicity and Possible Zoonosis

There is an obvious relationship between the Trypanosomatidae which infect invertebrates and plants and those which express pathogenicity for higher vertebrates (i.e., *Leishmania* spp. and *Trypanosoma* spp.). This has prompted speculation and some experimentation toward determining the disease-producing capacity of the former group. Thus, Lafont (155), the original discoverer of *Phytomonas*, thought that *Chamaesyce hypericifolia* became diseased when infected with *P. davidi*. Some authors (77, 107, 263) have been convinced that this same organism produces disease in *Euphorbia* spp. of Portugal, whereas others (127, 163, 277) have been unable to demonstrate signs of disease in plants infected with *Phytomonas*.

Holmes (123) thought that *Asclepias syriaca* parasitized with *P. elmassiani* exhibited symptoms of disease but concluded later (124) that the condition was common in autumnal die-back of the plant. McGhee (unpublished data) has never found any evidence of disease in hundreds of infected Asclepiadaceae examined. The only symptom in the plants is the disappearance of starch granules from the latex. The infection is confined to the laticiferous tubules of these two plant families. The function of the tubules is not known; some authors think that they are merely storage receptacles. If this is so, the likelihood of *Phytomonas* producing any observable pathology is remote.

Clearer evidence of pathogenicity was advanced in 1931 by Stahel (253), who associated *P. leptovasorum* with phloem necrosis of coffee in Surinam. Originally suspecting bacteria or fungi as the causative agents in *Coffea liberaca*, he described instead the promastigotes of a hitherto undescribed species of phytomonad in the

sieve tubes of infected plants. He was not able to culture the organism or to find its vector. The disease is so serious now that in the absence of any effective treatment, the affected area of Surinam is for all practical purposes a disaster area (R. B. McGhee and A. H. McGhee, unpublished data). The incubation period for the disease is probably 5 months.

Symptoms of infected trees include initial yellowing of the basal leaves, followed by yellowing of all leaves; as the roots begin to die back, the trees eventually die. Multiple divisions of the cambial cells occur, and abnormal phloem elements appear next to the wood cylinder. Stahel (253) described this condition as "multiple division of the phloem."

More recently, Parthasarathy (205), investigating the condition known as lethal yellowing of coconut palms, discovered *Mycoplasma* in diseased trees in Florida. Other investigators (219) have reported the presence of this microorganism in diseased palms in Jamaica. Investigations by Parthasarathy and his colleagues (206) in Surinam on trees similarily affected disclosed the presence of *Phytomonas* in the sieve tubes. The disease first described by Drost (63) as "Hartrot" is severe; most plants succumb to infection within 2 weeks after the appearance of the yellowing symptoms. Similar parasites were discovered in the oil palm *E. guineensis* in Peru (61) and later by Parthasarathy in Surinam. Symptoms in this tree were as severe as those in the coconut palm. Parasites were present in great numbers in the sieve tubes and were in all probability plugging the sieve plates of the sieve tubes, which are necessary for conducting nutritive material.

As is true of *P. leptovasorum* infections, no effective treatment is known. Reports of infected trees from Trinidad and Tobago have been verified (10). Thus, lethal yellowing of palms in Florida and Jamaica is associated with *Mycoplasma*; no *Phytomonas* has been found in trees from these locations. On the other hand, in Surinam, Colombia, Ecuador, and Peru, lethal yellowing is associated with *Phytomonas*, and no *Mycoplasma* has been found.

Although there have been various claims of infection and/or pathogenicity of vertebrates with members of the lower trypanosomatids, none has actually survived rigid scrutiny. In the early part of this century there were reports of infections of birds (72) and mammals (160, 161, 171, 172). One by one these claims have been refuted.

In 1957 McGhee (174) reported infection of chicken embryos with *Crithidia* isolated from the hemipteron *Euryophthalmus davisi*. Extensive growth and multiplication were obtained, first on the chorioallantoic membrane and then in the chorioallantoic cavity. A prerequisite for infection was the reduction of the ambient temperature of the host from 37 to 30°C. No pathology was observed, although choanomastigotes were observed within phagocytes in embryonic membranes. If *Crithidia* were maintained in embryos incubated at the lower temperature for 2 days, it was possible for the parasite to continue growth after the temperature of incubation was raised to 37°C. Later (236), it was found that five other species of *Crithidia* were capable of developing in the chorioallantoic fluid, as were *L. leptoglossi* and *B. euschisti*. *Crithidia* sp. from the yellow jacket (*V. squamosa*) flourished in chicken embryos incubated at 37°C without any adaptation to the higher temperature.

Daggett et al. (51) obtained evidence of infections of lizards and mice with *Herpetomonas megasaliae*.

A possible infection of a human with *Herpetomonas* has been investigated recently (R. B. McGhee and D. L. Reason, unpublished data). In June 1974 a female patient was admitted to Spohn Hospital in Corpus Christi, Tex., with rather ill-defined symptoms, including occasional periods of unconsciousness. Immediately thereafter, there was a febril period, which cleared spontaneously. A complete examination included a needle biopsy of the liver. A bit of the material was expressed into a culture tube containing a commercial preparation (Difco) of Balamuth medium. Nothing appeared until 7 days; no serious pathological changes were noted in the material preserved for sectioning and staining, with the exception of possible incipient cirrhosis. At the end of 7 days flagellated protozoa were observed in the tube containing Balamuth medium. The patient was later released from the hospital.

An examination of the culture indicated that the organisms were neither promastigotes (completely), which might have indicated leishmaniasis, nor epimastigotes, which would have been typical of Chagas' disease, which exists in Corpus Christi; rather, the organisms were a *Herpetomonas* sp. (Fig. 3), a protozoan thought to be parasitic strictly in Diptera.

Of the 10 valid species of *Herpetomonas*, all except two inhabit Diptera. One, which occurs in the hemipteron *Notonecta*, is doubtful. Since 1966, a species from a sawfly (Hymenoptera), one from *Megaselia scalarus*, and one from a reduviid hemipteron have been described (51, 254).

Inasmuch as the genus is considered a monoxenous symbiote of Diptera, the question to be considered was whether it somehow arose as a

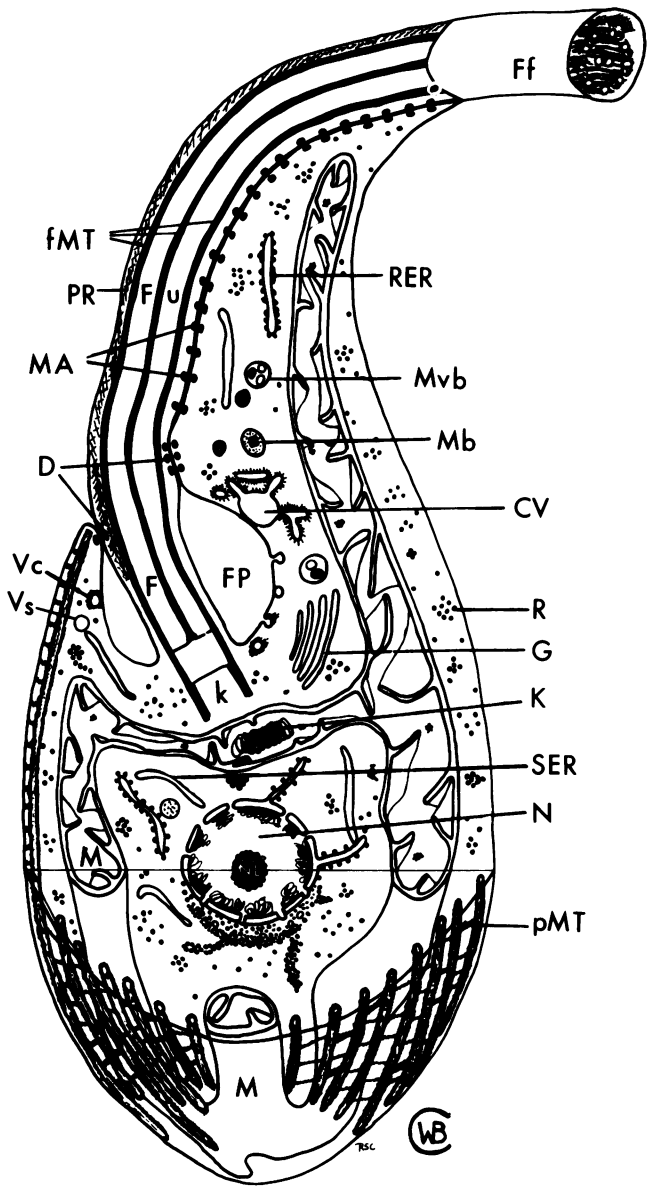

FIG. 3. *Diagram of an epimastigote. The anterior portion is shown in longitudinal section, and the tip of the flagellum is shown in oblique section (based on references 14, 17, 21, 22, 166, 167, and 208–212). CV, Contractile vacuole and spongiome tubules; D, desmosomal junction; F, flagellar pocket region of flagellum; Ff, free flagellum; Fu, undulating membrane region of flagellum; FP, flagellar pocket; G, Golgi apparatus; K, kDNA network in kinetoplast; k, kinetosome; M, mitochondrion; MA, maculae adherentes; Mb, microbody; fMT, flagellar MT; pMT, pellicular MT; N, nucleus; NL, nucleolus; PR, paraxial rod; R, ribosomes; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; Vc, coated vesicle; Vs, smooth vesicle; Mvb, multivesicular body.*

contaminant of the medium or from the liver of the patient. The commercial Balamuth medium contains no antibiotics, and none was added at the hospital. Therefore, if the medium had been contaminated by either feces or whole flies, the possibility of the survival of the herpetomonads would have been extremely remote. However, to the extent it was possible, both possibilities were considered.

For the parasite to survive in the milieu of a

human body, it had to be capable of surviving human body temperature. The suspect organism, designated HP, was inoculated into the chorioallantoic cavities of chicken embryos. In addition, two new isolates, *H. megaseliae* from *M. scalaris* and *H. muscarum* from *M. domestica*, were also used. Of the 12 embryos, 6 were held at 30°C and 6 were held at 37°C. Development occurred in all. Growth of the suspect organism was maximal at 37°C, whereas the other parasites grew best at 30°C.

A total of 10 embryos were injected intravenously with 0.05 ml of twice-washed herpetomonads and incubated at 37°C. All embryos survived until they were examined 8 days later. Blood films and impressions of the livers and spleens failed to reveal parasites. A pool of the livers was macerated, and a bit of material was placed in the regular diphasic medium. One of the four tubes inoculated became positive 7 days later.

Growth in cultures consisting of Cowperthwaithe medium buffered at pH 5 and 7.9 proved inconclusive. Maximum numbers were attained on day 2 or 3 in the case of *H. megaseliae*, between days 3 and 6 in *H. muscarum*, and on day 11 in HP.

Serum obtained from the human patient failed to agglutinate any of the isolates at any dilution.

Adult male White Leghorn chickens were immunized by intravenous injection with 9×10^6 to 11×10^6 cells of HP and *H. megaseliae*. Three injections of approximately the same number of parasites were made for 3 weeks. After 10 days test bleedings were made, and serum was harvested from each of the two chickens injected. *H. megaseliae* reacted with homologous antiserum at 1:640 and with *H. muscarum* at 1:640, but not at all with HP. Antiserum to HP reacted 1:640 with homologous antigen and at 1:160 with *H. megaseliae*, but not at all with *H. muscarum*.

The ability of all herpetomonads to exist at 37°C without previous adaptation, as was necessary for *Crithidia*, is surprising. The ability of HP to survive for 8 days in the livers of embryos at 37°C indicates an ability to exist in this habitat. All tests indicated that this *Herpetomonas* was not too closely related to either *H. muscarum* or *H. megaseliae*. The most common flies in both homes and hospitals are *Drosophila*, *Musca*, and *Megaselia*. Quite obviously, HP is from neither muscids or megaseliads. The latter has been known to breed in feces, shoe polish, formolized meat, and human cadavers. In addition to being the verified cause of eight cases of human myiasis, there is an authenticated report of adult flies in the feces of a human male (207). Although we have been unable to culture the species from *Drosophila*, the habitat of the duodenum, bile duct, and liver might be more conducive.

Although there is no proof of lower trypanosomatids infecting vertebrates, the possibility exists and should be considered by attending physicians and veterinarians.

CELL BIOLOGY AND PHYSIOLOGY OF THE LOWER TRYPANOSOMATIDS

Until recently, the small size of all trypanosomatids and their recalcitrance to standard methods of homogenization and fractionation made us dependent entirely upon electron microscopy of thin-sectioned material for information about cell structure. Our knowledge of the general ultrastructure of these cells is summarized in Fig. 3. Ideas about functions of these structures have rested largely on analogies with cells of higher eucaryotes. Now we are beginning to see the development of methods of homogenization, separation, and purification of subcellular components appropriate to these organisms, with a resulting rapid development in our understanding of some of these components.

Cell Surface and Plasma Membrane

In all trypanosomatids, the cytoplasm is delimited by a plasma membrane, a unit membrane of typical trilaminar appearance and dimensions, which extends down into the flagellar pocket and is continuous with the limiting membrane of the flagellum. Brooker (19) showed that in cells of *C. fasciculata* from the hindgut of a mosquito host, this membrane is covered by a layer of electron-dense, filamentous material giving a positive reaction for polysaccharide. This layer is absent from the membranes of the flagellum and the flagellar pocket. It is also absent from cells from the foregut and from culture forms. He suggested that there is an inherent ability to form cell coats, which is expressed only under appropriate environmental conditions; such a situation has been described for mammalian trypanosomes by Vickerman (278), where the architecture of the cell coats is markedly different. There has been no other systematic study of the occurrence of cell coats in lower trypanosomatids, but a careful examination of published micrographs of other species suggests that the descriptions and interpretations of Brooker are broadly applicable.

Internal to the plasma membrane lies an array of microtubules (MTs); although these structures are not in a strict sense elements of the plasma membrane, they are described here because they have been used as markers of the

plasma membrane in cell fractionation and as indicators of the sidedness of resealed vesicles formed by fragments of membrane and because they affect the distribution of the intramembranous particles of the plasma membrane. Angelopoulos (3) provides a comparative study of the arrangements in various genera, and Brooker (18) provides a detailed description of them in *C. fasciculata*.

The pellicular MTs lie in a clear zone of cytoplasm which is the equivalent of the ectoplasm of other protozoa; at regular intervals, connections occur between each MT and the plasma membrane overlying it and between adjacent MTs (60, 166, 212). The nature of these connections is uncertain, but they seem not to be the equivalent of the dynein arms of ciliary and flagellar MTs. Each tubule is constructed of the usual 13 protofilaments, and it is assumed that the fundamental subunits are tubulins, although apparently this has never been demonstrated directly. The MTs traverse the length of the body in parallel, straight or spiral paths (18, 134); as the diameter of a cell decreases posteriorly, some of the tubules end abruptly, so that the spacing between them remains relatively constant. Anteriorly, many of the MTs end near the opening of the flagellar pocket; the remainder reverse direction to course beneath the membrane of the flagellar pocket for some distance. Spacing between MTs in this region remains relatively constant because of reductions in diameter resulting from decreases in the diameters of the lumina rather than because of decreases in the number of MTs.

The pellicular MTs probably represent the trypanosomatid equivalent of the cytoskeleton of other eucaryotic cells, maintaining the characteristic body form while allowing flexibility and transient changes in shape and serving to anchor intrinsic membrane proteins. Although arrays of filamentous material have been described in association with specialized regions of the plasma membrane (14, 16, 17, 20, 21), there has been no systematic investigation of microfilaments and their relationship to these MTs, and the literature is devoid of information on actin- and myosin-like proteins in trypanosomatids. Such microfilaments, as well as the pellicular MTs, could provide the mechanism for the changes in body form and distribution of organelles which characterize the different phases of the life cycles of many trypanosomatids.

Although there are several analyses of total cellular lipids, their syntheses, and factors controlling their relative amounts (25, 50, 146, 148, 182, 203, 204, 265, 266, 269, 295), Hunt and Ellar (130) have provided the only extensive analysis of the plasma membrane. They prepared a membrane fraction from *Leptomonas collosoma*, which consisted of nearly pure plasma membrane, as judged by the presence of the characteristic pellicular MTs; the fraction contained 58% protein, 29% lipid, 8.2% carbohydrate, and 1.3% nucleic acid. On a weight percent basis, the lipids were about equally distributed among neutral lipids, phospholipids, and "acetone-eluting" lipids. The major phospholipids were phosphatidylethanolamine and phosphatidycholine, and unsaturated fatty acids constituted about 65% of the total fatty acids, with 18:1 as the major constituent. In agreement with data for other species, the only sterol was ergosterol, which amounted to about 25% of the membrane lipids. (The report [146] that cholesterol occurs in the lipids of *C. fasciculata* when this organism is grown in serum-containing media but not when it is grown in other media suggests that the detailed lipid composition may be significantly determined by the presence of preformed lipids in the environment.) Glucose was the major carbohydrate, with mannose and galactose occurring in equal amounts at about one-half the abundance of glucose; small amounts of xylose occurred. Proteins were not characterized, and percent content of the membranes included the pellicular MT protein. The membrane fraction was enriched 8- to 10-fold in acid phosphatase and in Mg^{2+}-dependent adenosine triphosphatase inhibited by Na^+ and K^+ but not by Ca^{2+} or ouabain. The reported twofold enrichment in 5-nucleotidase is not consistent with a primarily membranous location of this enzyme.

Linder and Staehelin (166) showed that the plasma membrane of this flagellate contains intramembranous particles randomly distributed over most of the cell body; the numbers of particles per square micrometer are not significantly different on the protoplasmic and external fracture faces, but the particles on the external face are significantly smaller. Occasional rows of particles, always on the protoplasmic face, were aligned with pellicular MTs. Because the particles in these rows are a random assortment of sizes and show no periodic spacing, Linder and Staehelin considered them to represent accumulations of particles of the protoplasmic face trapped by the membrane-MT linkers, which themselves do not project far enough into the bilayer to be revealed by freeze etching. Other organized arrays of intramembranous particles occur on the membrane fold of the flagellar pocket associated with the MTs and in the basal region of the flagellar membrane. Other areas of the pocket show a significantly greater density of intramembranous particles on the proto-

plasmic face; in the flagellar membrane, the greater density occurs on the external face. A less detailed analysis of *Herpetomonas samuelpessoai* (59) gave similar results, except that no rows of particles were seen and protoplasmic face and external face particles were not significantly different in size.

Cosgrove and Hanson (43) isolated a galactose-containing polysaccharide from *C. fasciculata* and tentatively localized it at the cell surface and in the flagellar membrane. Gottlieb et al. (90, 91) have shown that the bulk of the polysaccharides extractable from these cells is associated with the particulate fractions of cell homogenates and consists of a polydisperse mannan with a mean molecular weight of 1.4×10^4 and an araban-galactan with a molecular weight of not less than 2×10^5. This latter polysaccharide was localized at the cell surface, but the location of the mannan was not specified. Brooker (19) showed that the outer leaflet of the whole plasma membrane of *C. fasciculata* gives a positive reaction for polysaccharide in electron microscopic cytochemical tests, even in those stages in which a cell coat is not demonstrable. Other studies of cell surface saccharides have used lectins as probes, and the conclusions drawn below are subject to the uncertainties discussed by Nicholson (196). Cosgrove (unpublished data) found many α-D-mannose and *N*-acetyl-D-glucosamine residues but no L-fucose residues in *Herpetomonas muscarum muscarum*, as judged by the minimal concentrations of lectins producing agglutination; *Herpetomonas muscarum ingenoplastis* had many L-fucose and few α-D-mannose and *N*-acetyl-D-glucosamine residues. In both subspecies, residues appeared to be uniformly distributed over the cell and flagellar surfaces. De Souza et al. (57) found concanavalin A binding sites restricted to the flagella of *H. samuelpessoai*; inhibition of concanavalin A-mediated agglutination by both sucrose and α-methyl-D-mannoside presumably indicated that both α-D-glucose and α-D-mannose residues were present. Cytochemical tests showed some concanavalin A binding over the whole cell surface, but presumably not enough to produce agglutination. The less specific periodic acid cytochemical test showed the presence of saccharide residues over the whole body surface; the nature of these residues should be probed with other, specific lectins. In later papers (58, 71, 247) it was shown that both the distribution and the abundance of concanavalin A binding sites were strongly influenced by conditions of cultivation and phase of growth cycle. Dwyer and Chang (64) showed that *Blastocrithidia culicis* is agglutinated by both concanavalin A and the fucose-binding lectin from *Lotus*; the relative numbers of the two types of binding sites, as measured by the minimal lectin concentrations which produced agglutination, depended on the presence or absence of the endosymbiote naturally found in this species. Gueugnot and co-workers (95, 215) found α-D-glucose, α-D-mannose, L-fucose, D-galactose, and *N*-acetyl-D-galactose residues on both the body surfaces and the flagellar membranes of *Crithidia luciliae*.

There have been few systematic studies of membrane transport in the lower trypanosomatids, although reports (23, 96, 226) concerned mainly with other aspects of the cell biology of these organisms have provided some insights into transport properties. Min (184, 185) showed that both *C. luciliae* and *Crithidia arili* have specific, saturable transport of monosaccharides. Active transport operated at low external concentrations, and facilitated diffusion operated at high external concentrations. Whether these were two different aspects of a single transport mechanism or two different mechanisms operating under different environmental conditions could not be determined from his data. He did not attempt to isolate putative carrier molecules. Rembold et al. (228) suggested that glucose and galactose share a common transport site in *C. fasciculata*, and Bunn et al. (23) suggested that inhibition of membrane transport of fructose, glucose, and glycerol by 2-deoxyglucose is the basis for its inhibition of growth and respiration in *H. samuelpessoai*. Rembold and co-workers (227, 228) reported that *C. fasciculata* has separate active transport systems for biopterin, folic acid, and riboflavin; they also reported that the system for biopterin is inhibited by cyanide and dinitrophenol and by increased concentrations of Na^+, but is stimulated by increased K^+. The absence of an ouabain effect was interpreted as evidence against linkage to Na^+/K^+ transport. Rembold and Langenbach (226) found that colchicine competitively inhibited the influx but not the efflux of biopterin; lumicolchicine inhibited the influx non-competitively. Colchicine did not penetrate the cells but bound in a non-saturable fashion to the membrane, perhaps by a general hydrophobic interaction with membrane lipids. They proposed that this incorporation of colchicine into the matrix of the membrane sterically interferes with the biopterin carrier sites in the membrane. The only systematic study of amino acid transport is the analysis by Midgley (183) of the uptake of α-aminoisobutyric acid, in which he showed that this amino acid is actively taken up by a system of broad specificity which does not involve symport or

cotransport of K^+ or Na^+ and operates under both aerobic and anaerobic conditions. He proposed as a working hypothesis an H^+ symport system in which an electrochemical gradient of protons across the cell membrane is maintained by an H^+-translocating adenosine triphosphatase in the membrane. This proposed mechanism would represent only one of several amino acid transport systems which are constitutive components of the membrane transport processes of the cell.

Steinert and Novikoff (256) first demonstrated the uptake of particulate matter in trypanosomatids and described a cytostome through which such uptake is mediated; previously it had been assumed that only diffusive and active transport processes occurred. It now seems probable that the ability to endocytose particulate matter and particle-free volumes of extracellular fluid is a general ability of these flagellates but that only the membrane of a portion of the flagellar pocket has that ability. Brooker (17) has described an extensive cytostomal system with supporting MTs in *C. fasciculata*; endocytosis of ferritin occurs by numerous vesicles formed from the cytostomal membrane. The cytostome may represent a mechanism for nonspecific endocytosis of particulate or soluble materials, and the coated vesicles associated with the membrane of the posterior half of the flagellar pocket may represent a mechanism for receptor-mediated endocytosis of specific molecules (87). Although so extensive a cytostome has not been described in other lower trypanosomatids, folds and grooves in the membrane of the flagellar pocket associated with groups of MTs, in a fashion reminiscent of the cytostome of *C. fasciculata*, occur in *L. collosoma* (166) and *H. megaseliae* (135). The junctional specializations in membranes of flagella and flagellar pockets at the rims of the pockets (see below) imply both restricted communication between the contents of a pocket and the extracellular environment and restricted flow of membrane constituents from the general body surface to the membrane of a pocket. The conditions under which pinocytosis is induced and maintained and its contribution to the economy of the cell deserve serious, systematic investigation.

There emerges from these scattered studies of trypanosomatid cell membranes the general picture of a structure conforming to the fluid mosaic model of cell membranes, with a sterol and unsaturated fatty acid composition consistent with fluidity over a wide range of temperatures, containing intrinsic membrane proteins and overlaid by a layer of extrinsic glycoproteins, the extent of development of which is controlled by environmental factors. Some of the intrinsic proteins are restricted to one or the other half of the lipid bilayer; others extend through the whole thickness of the membrane. These proteins have transport, recognition, and junctional functions, and at least some of them are glycoproteins. Considerable regional specializations in the concentration and array of these proteins occur and are maintained and perhaps produced by anchoring filaments and MTs in the underlying cytoplasm. There is no information on the possibility that comparable regional specializations in the lipid organization occur or on possible alterations in lipid composition with environmental temperature. A comparison of strains or species of *Crithidia* capable of growth at 37°C (67, 232, 233) with those restricted to temperatures below 30°C should test the adequacy of these organisms as models for *Leishmania* and *Trypanosoma*, in which the life cycles may require existence at sharply different temperatures.

Cytoplasmic Organelles

There have been few systematic studies of cytoplasmic organelles other than the kinetoplast-mitochondrion complex, and most of the following material has been gleaned from studies of electron micrographs published to illustrate aspects of kinetoplast-mitochondrion structure and condition. Brooker (17) and Brun (22) have provided detailed and extensive studies on the ultrastructures of *C. fasciculata*, *C. luciliae*, and *H. muscarum*.

Electron micrographs show that the cytoplasm is crowded with ribosomes; some of these are free, whereas others are grouped together to form polysomes or attached to the outer nuclear membrane or portions of the endoplasmic reticulum. These cytoplasmic ribosomes have sedimentation coefficients of 80S and dissociate into large and small subunits of 58S to 60S and 40S to 41S. In *C. oncopelti*, dissociation occurs especially readily when protein synthesis is not occurring or when the concentration of Mg^{2+} is lowered. The subunits formed unfold to 45S and 33S configurations which are stable. Ribosomal ribonucleic acids (RNAs) have molecular weights of 0.74×10^6 to 0.84×10^6 and 1.30×10^6 to 1.38×10^6; the small RNA is stable during electrophoresis under denaturing conditions, but the large RNA dissociates into components of 0.70×10^6 and 0.56×10^6, 0.83×10^6 and 0.56×10^6, or 0.73×10^6, and 0.57×10^6 daltons, depending on the species (48, 187, 188, 250). The dissociation is attributed to a "hidden break" (223, 250). Gray (93) identified four minor, alkali-stable dinucleotides from ribosomal RNA of *C.*

fasciculata; three of the constituent nucleosides are known from other ribosomal RNAs, but one, O^2-methylinosine, is entirely novel. Although these dinucleotides were isolated from total cellular ribosomes, it is unlikely that they are derived solely from mitochondrial ribosomes, which are estimated to constitute about 1% of the total cellular ribosomal RNA. The relatively large size of the smaller ribosomal RNA and the lability of the large RNA also set these flagellates apart from most eucaryotes except *Euglena gracilis* and *Acanthamoeba castellani*, so far as our scanty and scattered sampling provides a basis for comparison. The messenger RNAs of the lower trypanosomatids have not been investigated.

Cytoplasmic protein synthesis has been little studied; it is effectively inhibited by all standard inhibitors of eucaryotic cytoplasmic protein synthesis when precautions are taken to see that permeability barriers do not prevent access of the inhibitor molecules to the sites of synthesis. Cell-free ribosomal systems from *C. fasciculata* depleted of intrinsic messenger RNA have been shown to direct the translation of synthetic polyuridylic acid (36), of polyadenylated *Crithidia* messenger RNA, and of rabbit α- and β-globin messenger RNAs (132). In this species, the AUG codon and methionine-transfer RNA, rather than formyl-methionine-transfer RNA, may be involved in the initiation of protein synthesis on the 80S ribosomes (131). Much more extensive and systematic study of RNA structure and protein synthesis in these flagellates is very desirable. The resulting information will help determine the extent to which protozoa diverge from higher eucaryotes in their fundamental biochemical processes and will provide a basis for developing realistic evolutionary relationships.

The endoplasmic reticulum is moderately developed and shows the usual unit membrane appearance and relationships with the outer nuclear membrane. Both smooth and rough types are present. The Golgi apparatus is also well developed and is located anterior to the nucleus and near the base of the flagellar pocket. Its morphology is typical (55, 135). The region surrounding the Golgi apparatus contains numerous coated and smooth vesicles, and an extension of the endoplasmic reticulum subtends it. Although there have been no experimental investigations of endoplasmic reticulum-Golgi apparatus function in these cells, the morphology and interrelationships observed in thin sections support a conventional role, but one that must be restricted to the formation of membrane constituents and lysosomal enzymes because these cells do not directly secrete extracellular proteins. One report (212) specifically notes the apparent absence of the Golgi apparatus.

Clark (37) and Hajduk (100) have shown that contractile vacuoles are of widespread but not universal occurrence. They are located near the flagellar pocket, into which they discharge at systole, and there is usually only one per cell. The contractile vacuole is surrounded by an extensive system of 60- to 70-nm tubules bearing coats of pegs on the cytoplasmic surfaces (17, 167). The membranes of these spongiome tubules may be continuous with the contractile vacuole membrane, and the general relationship suggests very strongly that the spongiome membranes carry out the actual segregation of the fluid expelled at systole. During diastole this fluid is transferred from the tubules to the growing contractile vacuole, which is permanently attached to the membrane of the flagellar pocket by an adhesion plaque. When maximum size of the contractile vacuole is reached, the tubules detach, and transient aqueous channels appear in the adhesion plaque, through which the contents of the contractile vacuole are expelled into the flagellar pocket (167).

The rhythmic activity of these contractile vacuoles is relatively slow compared with the vacuoles of free-living protozoa, and they probably account for the relatively high resistance to osmotic lysis that many species of these flagellates show. Cosgrove and Kessel (44) showed that contractile vacuole output in *C. fasciculata* is proportional to the calculated osmotic pressure gradient across the cell membrane over a considerable range of osmotic concentrations in the medium but that regulation of cell volume and of internal osmotic pressure under such conditions is very imperfect. This imperfect regulation and the ability of these flagellates to survive and multiply in media so dilute as to produce nearly spherical cells or so concentrated that the cells are flattened and leaflike raise the question of whether these vacuoles are primarily osmoregulatory or whether any osmoregulatory effect is incidental to some other primary function, such as ion regulation. Failure to demonstrate in plasma membrane fractions an Na^+/K^+ adenosine triphosphatase inhibited by ouabain (130; W. B. Cosgrove and D. Stone, unpublished data) implies that Na^+/K^+ regulation is not accomplished by the general cell surface. Some species of bloodstream trypanosomes cannot develop contractile vacuoles when placed in dilute environments; it would be interesting to determine whether the insect and culture forms of these species also lack this ability.

Other common cytoplasmic structures are bundles of microfilaments, a variety of mem-

brane-bound vesicles in addition to those already described, multivesicular bodies, and electron-dense, compact membrane-bound bodies. A few species also contain intracellular symbiotes (see below). The function and composition of the bundles of microfilaments not associated with membrane specializations are not known. Some of the membrane-bound vesicles are commonly described as lipid droplets, but the inability of trypanosomatids to metabolize triglycerides makes it difficult to understand why such accumulations of lipid should be formed. Multivesicular bodies consist of vacuoles, each containing several to many smaller vesicles suspended in a clear matrix. Configurations occur which suggest either constriction into two smaller multivesicular bodies or fusion of two such bodies. The demonstration of acid phosphatase in the intervesicular space suggests that these bodies are heterolysosomes. It is uncertain whether lysosomes in the conventional sense occur in these flagellates. Cytochemical tests demonstrate acid phosphatase (a marker enzyme for lysosomes) only in these multivesicular bodies and the contents of the flagellar pocket; attempts to determine the distributions of this enzyme and other acid hydrolases between soluble and particulate fractions of homogenates have given conflicting results. It seems likely that these conflicts resulted from methods of homogenization and fractionation, which produced varying degrees of damage to the membranes of subcellular particles, and that the problems will not be finally resolved until better techniques are developed. In the meantime, the hypothesis of Brooker (17) is useful. He has hypothesized that multivesicular bodies are formed by the fusion of pinocytotic vesicles and lysosomes, that the digestible contents of the pinocytotic vesicle are hydrolyzed and absorbed through the membrane of the multivesicular body, and that the indigestible material and lysosomal enzymes remaining are ultimately discharged into the flagellar pocket by fusion of the membranes of the pocket and the multivesicular body. This hypothesis provides an explanation for the acid phosphatase activity and particulate debris in the contents of the flagellar pocket.

The most abundant electron-dense, membrane-bound bodies are usually identified as microbodies. In their electron microscopic morphology they resemble the microbodies or peroxisomes of higher eucaryotes (including the frequent occurrence of crystal-like inclusions), but the experimental evidence in favor of this identification is sparse (53, 60, 192). Although these bodies give positive cytochemical reactions for catalase, cell-free homogenates in which they are well preserved usually have little or no sedimentable catalase activity; attempts to demonstrate hydroxy acid and D-amino acid oxidases have given conflicting results. In the bloodstream forms of the *T. brucei* complex trypanosomes, which lack cytochrome-mediated respiration and catalase activity but have many microbody-like organelles, it had long been thought that the particulate glycerophosphate oxidase system responsible for oxygen consumption was located in the microbodies (110, 189); by analogy, a similar function was attributed to the microbodies of lower trypanosomatids, even though their oxygen consumption is largely cytochrome mediated. However, Opperdoes et al. (200) have shown conclusively that this oxidase system is located in the mitochondria of *T. brucei* and that none of the usual marker enzymes can be demonstrated in its microbodies. The only enzyme detected was oxidized nicotinamide adenine dinucleotide-linked glycerophosphate dehydrogenase; these authors also demonstrated this enzyme and catalase in the microbodies of *C. luciliae*. Nonetheless, the conclusion of Müller (189) ("It is still impossible to define the biochemical nature of microbodies in lower trypanosomatids and their relations to other types of microbodies") continues to be a valid summary of our understanding of them.

Kinetoplast-Mitochrondrion Complex

The presence of a kinetoplast is diagnostic of the order Kinetoplastorida, to which trypanosomatids and bodonids belong. Originally described as a strongly eosinophilic granule at the base of the flagellum in cells stained with Romanowsky-type stains, the kinetoplast is now known to be an enlarged and specialized region of a mitochondrion containing a complexly organized network of kinetoplast DNA (kDNA), amounting to as much as 20% of the total cellular DNA and representing the equivalent of all of the mitochondrial DNA of other cells. The kinetoplast envelope consists of two membranes which are continuous with the inner and outer membranes of the rest of the mitochondrion. The inner membrane bears cristae in forms with normally developed mitochondria, and the finely granular matrix contains ribosome-like objects which are smaller and much less concentrated than the cytoplasmic ribosomes. Paulin (210, 211) showed conclusively the unitary nature of the chrondriomes of *B. culicis*, *C. fasciculata*, and *T. cruzi*; each cell contains a single mitochondrion consisting of several interconnected tubular elements ramifying throughout the cell. The kinetoplast is an extension from one of these elements or a cross-connection between two of them. In *B. culicis*, but not in the other species, these tubular elements are closely appressed to

the plasma membrane; in these regions the pellicular MTs are absent.

Because changes in the extent of development of mitochondrial morphology and function and in the location of the kinetoplast are characteristic of the life cycles of *Leishmania* and *Trypanosoma* and because exposure to certain drugs which are trypanocidal produces alterations in the kDNA, the kinetoplast has been the object of intensive investigation, and there has been rapid progress in our understanding of the organization of kDNA (35, 142–144, 159, 224, 239, 242). It is now generally agreed (13) that this DNA typically consists of two types of covalently closed circular molecules—minicircles of 0.2- to 0.8-μm contour length and maxicircles of 10 to 12 μm. Contour lengths of both types of circles are constant within a species but not among species. There is no protein associated with either type of circle (248). Small numbers of molecules interpreted as fused dimers of minicircles also occur and may be intermediates in recombination. In *C. fasciculata* but not in *C. luciliae*, heterogeneous circles intermediate in size between mini- and maxicircles occur; their significance is not known. In *H. muscarum ingenoplastis* minicircles are absent, and the kDNA, which does not show the typical ultrastructural network organization in situ (284), contains catenated large circular molecules (S. L. Hajduk, personal communication). The linear DNA occasionally reported (194, 259) is now generally interpreted as an artifact (i.e., contaminating nuclear DNA or linearized circles).

Before the occurrence of maxicircles was known, it was difficult to understand how the kDNA could encode any significant amount of genetic information because of the small size of the minicircles; proof of the presence of maxicircles, which are comparable in size to mitochondrial DNA molecules (141, 259), and the demonstration that only they are transcribed in *P. davidi* (35) and *C. luciliae* (122) remove the necessity for ascribing an informational function to the minicircles. The rapid development of sequence heterogeneity in minicircles and the highly conserved sequences of maxicircles are consistent with the interpretation that only the maxicircles have an information function. Borst and Hoeijmakers (13) discuss various suggested functions for the minicircles, which represent at least 95% of the kDNA present in a network.

However, it has still not been proven that the maxicircles play a conventional mitochondrial DNA role. Their transcription has been demonstrated (35, 113, 122, 244, 245), but translation of the transcription products is uncertain. Amman (2) attempted to use kDNA from *C. fasciculata* as a template in two cell-free transcription-translation systems widely used in studies of procaryotic protein synthesis. Despite a systematic search for optimum conditions of ionic concentration and other factors, her results failed to show a consistent, statistically significant increase in incorporation of [^{3}H]leucine into acid-precipitable material in the presence of kDNA compared with controls from which kDNA was omitted. Although some of the labeled material produced in vitro was electrophoretically indistinguishable from material isolated from kinetoplasts after in vivo labeling, immunological methods necessary to demonstrate identity were not applied. However, these negative results cannot be taken to show inability of kDNA to direct the synthesis of characteristic proteins in vivo. In addition to the well-known difficulty of demonstrating mitochondrial DNA function in vitro (12), these results might also have resulted from inadequate or insensitive methodologies or from the use of kDNA badly damaged in preparation. Zaitseva and associates (292) have reported that the kinetoplast in *C. oncopelti* contains a characteristic DNA-dependent RNA polymerase and its own protein-synthesizing system, that isolated kinetoplasts incorporate [^{14}C]leucine into acid-insoluble material, and that kinetoplast ribosomes function in cell-free protein-synthesizing systems. They did not compare the products of in vitro syntheses with the compounds produced in vivo; their success in demonstrating in vitro synthesis may be due to their use of homologous systems. Their conclusion that "kDNA codes only one or several protein factors which regulate the formation of a variety of kinetoplast proteins coded for by the nuclear genome" unfortunately derives from the belief that the only components of kDNA are minicircles and linear DNA molecules "probably composed of nucleotide sequences of simple circular molecules repeating themselves many times." Indirect demonstration of kDNA function by using specific inhibitors of mitochondrial and cytoplasmic protein syntheses has also been unsuccessful. Except for the controversial report of Laub-Kupersztejen and Thirion (140, 157), in vivo protein synthesis, whether measured by incorporation of labeled amino acids or by growth of cells, seems not to be detectably inhibited by chloramphenicol; however, whether these results indicate a true absence of kDNA-directed protein synthesis, failure of chloramphenicol to reach or to react with the sites of translation within the kinetoplast, or merely a kinetoplast fraction of total protein synthesis which is too small to be detected cannot be decided.

Replication of kDNA is semiconservative, and ^{3}H-labeled kDNA is equally distributed to

daughter cells through at least five generations (245). Using pulse-labeling, Simpson and associates (240, 246) showed that replication in *C. fasciculata* begins at two sites 180° apart on the rim of the kDNA network and progresses around the rim; newly replicated DNA is gradually displaced toward the center. Englund (68, 69) recognized three types of networks: (i) form I, having a high buoyant density and containing only covalently closed minicircles; (ii) form II, banding at low density and containing only gapped or nicked minicircles; and (iii) replicating networks of intermediate density and containing both types of minicircles. Replication of form I networks begins at the periphery; replicated minicircles remain nicked or gapped, and as replication proceeds, this peripheral zone of such minicircles enlarges centripetally until only nicked or gapped circles are present and the network is form II and double the size of form I. Replication is completed by the covalent closure of all minicircles. The maxicircles form only 3 to 5% of the mass of the network, and their replication could not be studied. In exponentially growing cultures, the relative amounts of the three types of networks is in reasonable agreement with the relative durations of the G_1, S, and G_2 phases of the cell cycle (46), assuming that separation of a form II network into a form I network occurs late in G_2. This separation must be very closely associated temporally with covalent closure of the minicircles, since no form I networks of double normal size were detected. Nothing is known about the mechanism of this separation. Some of the numerous electron micrographs in the literature show configurations of the network, suggesting separation of the disk by a constriction through its center; other micrographs suggest an equatorial separation into two parallel disks which then slide apart. Nothing is known about the membrane events which result in the formation of two separate kinetoplasts.

Werbitzki (287) reported in 1910 that when bloodstream trypanosomes were exposed to certain dyes, some or all of the cells lost their kinetoplasts. Production of such "akinetoplastic" cells also occurs in lower trypanosomatids (42, 99, 255), and populations of several species in culture contain small percentages of such individuals, which are produced spontaneously (45). Although no kinetoplasts have been detected by light microscopy of Romanowsky-stained preparations, Trager and Rudzinska (272) showed that in *Leishmania tarentolae* this akinetoplastic condition is characterized by the loss of the typical network organization of the kDNA rather than by its complete absence; they introduced the term dyskinetoplastic as more suitable for this condition. However, Hajduk (102) has shown that the dyskinetoplastic state as diagnosed by light microscopy may in fact be either (i) a disorganization and fragmentation of the kDNA network, or (ii) a complete absence of kDNA. In the first case, the kDNA is not detectable by phase-contrast microscopy or by Romanowsky staining but can be detected by fluorescence and electron microscopy and by analytical ultracentrifugation; there is little or no reduction in kDNA content per cell. In both cases, the membranes of the kinetoplast persist, but cristae may be reduced or absent. The production of the dyskinetoplasty requires cell division and results from interference with replication of kDNA so that it is gradually "diluted out" (170, 255), or kinetoplast replication is so delayed that only one kinetoplast is present when cell division occurs, with the result that one daughter cell receives no kinetoplast (42, 102). Dyskinetoplastic cells of lower trypanosomatids, however produced, have never been maintained in culture, but their ability to undergo a few cell divisions suggests that this failure is probably the result of inadequate cultural conditions, an interpretation reinforced by the ability of dyskinetoplastic bloodstream trypanosomes to propagate indefinitely in their vertebrate hosts.

Comparisons of normal and partially dyskinetoplastic populations of *C. fasciculata* and of mitochondria isolated from them have been pursued as possible approaches to elucidating kinetoplast or kDNA function (112, 170). Although dyskinetoplastic cells always show a reduction or complete absence of cristae in the kinetoplast mitochondrion, there are no other obvious changes in morphology. The impossibility thus far of obtaining 100% dyskinetoplastic populations has prevented comparisons of the compositions of the mitochondrial membranes of normal and dyskinetoplastic cells. Partially dyskinetoplastic populations show a reduction in rate of oxygen consumption, in activity of mitochondrial enzymes, such as succinic dehydrogenase, and in cytochrome content proportional to the percentage of dyskinetoplasty. If it is assumed that treatments which produce dyskinetoplasty affect only the kinetoplast genome and never the nuclear genome, then these observations are consistent with a role for kDNA in mitochondriogenesis, but specification of the details is not warranted. The proper integration of mitochondrial DNA-directed peptide subunits into functional units in other eucaryotes requires the coordinate syntheses of other subunits of both mitochondrial and cytoplasmic origins (12) and presumably the proper pattern and rate of synthesis of membrane lipids. Even the validity of

the assumption that the treatments used affect only kDNA is doubtful; the most frequently used drugs are known to bind to both nuclear DNA and kDNA, with preferential binding to the latter.

Both the kinetoplast region and the main body of the mitochondrion have the usual double membrane structure. The inner membrane of both regions is thrown up into cristae. In the main body of the mitochondrion, these cristae may be platelike or villous and often show complex configurations of parallel foldings, concentric whorls, or tubules. Brooker (17) shows 11-nm projections from the platelike cristae. The mitochondrial matrix is finely granular and contains electron-dense bodies morphologically like the calcium-rich bodies in the mitochondria of higher eucaryotes. There is no evidence for mitochondrial DNA outside the kDNA network in normal cells. In *T. brucei* complex trypanosomes, the bloodstream forms show poorly developed inner mitochondrial membranes, absence of a functional cytochrome system, and terminal oxidation mediated by the glycerophosphate oxidase system. Insect and culture forms of the same species show well-developed inner mitochondrial membranes and terminal oxidation mediated largely by cytochrome systems. In these respects they resemble most of the lower trypanosomatids, which thus have been of interest as more easily investigated models.

Early attempts to obtain mitochondrial preparations showing good respiratory control were usually unsuccessful; it is obvious now that the unitary nature of the mitochondria of these cells probably makes it impossible to isolate and study mitochondria with intact, undamaged membranes. However, Toner and Weber (270) have obtained preparations from *C. fasciculata* which do show good control, although they obviously consist of resealed fragments of the original mitochondria. Even with such preparations, results must be interpreted cautiously; no matter how rapid and perfect the resealing of the membranes of the fragments may be, it is unavoidable that there will be some loss of the soluble components of the matrix and intermembranous space and possibly also some loss of the less firmly bound membrane components. Some of these lost components would play regulatory roles in situ. Nonetheless, the various published reports can be taken to indicate in general that when a mitochondrion has typical ultrastructure, it also has typical enzymatic activity, including Krebs cycle, cytochrome electron transport, and oxidative phosphorylation systems (56, 110, 111, 114, 153, 154, 170, 201, 270). Oxygen consumption is nearly or completely abolished by cyanide and other classical inhibitors of the cytochrome system, and low-temperature difference spectra show the presence of cytochromes *c*, *b*, aa_3, and possibly *o* (65, 116, 149, 151, 152, 251). Whether cytochromes aa_3 and *o* both function as terminal oxidases is uncertain. Kronick and Hill (149) found evidence for a functional role of cytochrome *o* in *B. culicis* but not in *C. oncopelti*, at least at relatively high oxygen concentrations. However, Srivastava (251) has described difference spectra which indicate that only cytochrome *o* is present in the early phases of growth of a culture and that it is replaced by cytochrome aa_3 in stationary-phase cells. In *C. fasciculata*, Hill and associates (114, 116) have variously reported spectral evidence and cyanide sensitivity consistent with a cytochrome *o*-type terminal oxidase and with only a cytochrome aa_3 type. Kusel and Storey, after a preliminary report of cytochrome *o* in this species, could find no evidence for it later in photochemical action spectra (152). Some of these contradictions seem to be the result of the use of more sensitive and specific techniques and of mitochondrial preparations instead of whole cells or unfractionated homogenates; for example, Kusel and Story found that some of the peaks in low-temperature difference spectra of whole cells were absent from mitochondrial preparations and were not due to cytochromes but possibly to heme-protein storage complexes or peroxidase-like hemoproteins. Other sources of confusion are the different strains (sometimes of dubious authenticity) of the same species and cells harvested from different phases of the culture cycle or from cultures grown under different conditions of aeration; this latter point is particularly important because of the possibility that the predominant functional terminal oxidase may be determined by the oxygen concentration of the medium during growth. It is apparent that in general most lower trypanosomatids have well-developed mitochondria, highly cyanide-sensitive respiration, and a cytochrome aa_3, *b*, *c* (and possibly *o*) electron transport system. It seems probable that two other characteristics can be added, the presence of an oligomycin-sensitive Mg^{2+}-adenosine triphosphatase and the presence of an adenine nucleotide translocator (201). The oligomycin-sensitive adenosine triphosphatase differs from the mammalian system in its insensitivity to aurovertin, and such differences are worth exploring as possible approaches to chemotherapy of trypanosomiases.

H. muscarum ingenoplastis may represent a unique and valuable case of a kinetoplast mutant in these lower trypanosomatids. Wallace et al. (284) discovered that its putative sister subspecies, *H. muscarum muscarum*, has a kinetoplast

and mitochondrion of typical ultrastructure in thin sections; however, the kinetoplast of *H. muscarum ingenoplastis* has only loosely arranged kDNA, and the mitochondrion lacks cristae. Hajduk (personal communication) has confirmed these differences and has in addition shown that *H. muscarum muscarum* has the molecular organization of kDNA and the mitochondrial functions typical of lower trypanosomatids. In contrast, *H. muscarum ingenoplastis* has cyanide-insensitive respiration inhibited by salicylhydroxamic acid, probably lacks cytochromes *b* and aa_3, and has no oligomycin-sensitive adenosine triphosphatase. Its kDNA consists in part of megacircles which have molecular weights of 11×10^6 and 15×10^6 and show extensive microheterogeneity.

Nucleus and Cell Division

The nuclei of trypanosomatids have a typical eucaryotic structural organization; the nuclear envelope consists of two unit membranes penetrated by numerous pores. Electron microscope images show that these pores may be open or closed and that material may be exchanged between nuclei and cytoplasm through them. The small sizes of the cells and nuclei have precluded any determination of nuclear envelope permeability in situ, so it is impossible to say whether this envelope has unusual physical and chemical properties. Internal nuclear structure is poorly preserved in the dried, methanol-fixed smears which are the usual basis for light microscopic descriptions of morphology. In wet-fixed preparations stained with appropriate cytological techniques, chromatin and DNA in interphase nuclei occur as an apparently finely granular material distributed over the inner surface of the nuclear envelope; an endosome or nucleolus composed mostly of RNA occupies the central region of the nucleus. Electron microscopy adds little to this description, except to confirm the granular nature of the nucleolus.

Preoccupation with the structure, organization, and significance of kDNA has resulted in nearly complete neglect of nuclear DNA. Leaver and Ramponi (162) report that nuclear DNA preparations from *C. oncopelti* contain 15 to 20% histone, 5 to 10% RNA, and 20 to 30% DNA; no mention is made of nonhistone proteins. The histones included components electrophoretically similar to those of calf thymus and two additional components very rich in lysine and alanine. The evidence of these authors refutes an earlier report that histones are absent and provides a basis for the nucleosome organization clearly shown by Borst and Hoeijmakers (13). There have been no reports of analyses of nuclear DNA for repetitive sequences or satellite DNA, but the consistent appearance of shoulders on the nuclear DNA peaks in optical density tracings of cesium chloride density gradient separations of whole cell DNA hints of its presence. Mol et al. (186) have reported a much lower frequency of $(dA\text{-}dT)_{20\text{-}25}$ tracts in *C. luciliae* than in the other primitive eucaryotes studied.

The nuclei of the flagellates fall into a size class too small for adequate study by light microscopy and too large for easy study by electron microscopy. As a result, there is only scattered information on nuclear division, mostly extracted from micrographs primarily intended to illustrate other features. The nuclear envelope persists during mitosis; the nucleolus persists in some species but not in all. The chromatin remains in its peripheral location, and no evidence of chromosomes, kinetochores, or chromosomal MTs can be seen, although spindle MTs are clearly visible. There is no evidence of centrioles or of kinetosomal involvement in mitosis. It seems likely that mitosis in these cells is more "primitive" than in higher eucaryotes. The absence of a complete description of mitosis in any trypanosomatid is a serious defect in our understanding of these flagellates. The use of fluorescence microscopy with sensitive DNA probes, such as DAPI (101) and mithramycin (109), in combination with serial thin-sectioning techniques on populations with a high degree of synchrony (66, 254; W. B. Cosgrove, M. J. Skeen, and S. L. Hajduk, J. Protozool., in press) should make possible a complete description of nuclear division in one of the easily cultured species. Such a description is necessary to identify how mitosis differs from mitosis in higher eucaryotes and to determine whether these flagellates, which do not have known sexual processes, are haploid or diploid.

Cosgrove and Skeen (46) described in detail the cell cycle in *C. fasciculata*; replication of kDNA and nuclear DNA occurs essentially simultaneously, with completion of kinetoplast division preceding completion of mitosis by 1.4 to 3.7% of the generation time, depending on temperature. Steinert et al. (261) reported similar results with *C. luciliae*, except that kinetoplast division is not completed until after mitosis. This latter situation should carry a higher probability of cell division producing one dyskinetoplastic daughter cell; it would be interesting to know whether *C. luciliae* has a higher percentage of spontaneously dyskinetoplastic individuals than *C. fasciculata*. The observations (42, 102) of divisions of dyskinetoplastic cells of *C. fasciculata* indicate that a normal kinetoplast is not essential for cytokinesis and suggest that

the temporal coordination between kinetoplast and nucleus in both DNA synthesis and division is maintained in nature by the inability of dyskinetoplastic cells to multiply under conditions suitable for normal cells.

Edwards et al. (66), using populations of *C. fasciculata* synchronized by density gradient centrifugation, showed that cellular dry weight and the amounts of protein and RNA per milliliter of culture medium increased continuously, doubling in one cell cycle; the rate of oxygen consumption, although showing an overall doubling, displayed complex oscillatory components, with a period of approximately 20% of the cycle length and an amplitude of 8 to 16% of the average cell respiration. Cyanide inhibition did not dampen or attenuate this oscillatory component. Complex changes in the pool sizes of adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate were temporally correlated with changes in the rate of oxygen consumption and not with changes in the patterns of biosynthesis of cell components. It is not apparent how these patterns, occurring during a single cell cycle, are related to changes in catabolism, which Marr et al. (171, 173) describe in unsynchronized populations as they proceed through the exponential growth phase.

Paulin (208) showed that recently divided cells of *C. fasciculata* have a prokinetosome lying parallel and close to the flagellum-bearing kinetosome. The first sign of approaching cytokinesis is a lateral migration of this prokinetosome and its enlargement, followed by the formation of the basal, intracellular portion of the new flagellum. Elongation then produces the new daughter flagellum. While these changes are occurring, new prokinetosomes are formed at right angles to the existing kinetosomes so that each daughter cell possesses one flagellum-bearing kinetosome and one prokinetosome; numerous electron micrographs of the kinetosomal region of other species suggest that this description is generally applicable.

At the light microscope level, the first sign of approaching cytokinesis is the appearance of the stub of the new flagellum in the flagellar pocket. Cosgrove (unpublished data) found that elongation of a new flagellum is accompanied by a shortening of the old flagellum until the two are the same length; thereafter, elongation occurs simultaneously in both. These observations suggest that recycling of tubulin subunits occurs from old to new flagella at first, but no studies of tubulin pools and synthesis have been made. Before flagellar elongation is complete, cytokinesis begins at the anterior end and proceeds posteriorly, as is typical of flagellates. Nothing is known of the mechanism of cytokinesis or of the possible role of microfilaments in it. At least in *C. fasciculata*, near the end of the process the daughter cells are joined at an expanded, disk-shaped region sharply delineated by phase microscopy of living cells; this region may be homologous to the contractile ring of metazoan cells. We know nothing about the mechanisms or scheduling of the duplication of other cytoplasmic structures. Our ignorance in these matters is rooted in the difficulty of studying them in unsynchronized populations containing only small percentages of cells in the desired stage; now the availability of new methods for synchronizing cultures (66, 254) removes that obstacle to progress.

Flagellar Structure and Activity

Except in dividing cells, trypanosomatids characteristically bear a single flagellum, but Rogers and Wallace (231) reported that biflagellated cells of *H. muscarum ingenoplastis* predominated in cultures and in an insect host. In all species, the flagellum arises from a kinetosome close to the kinetoplast, courses through an invagination of the cell surface (the flagellar pocket), and except in amastigotes emerges as either a free flagellum or an undulating membrane coursing along the surface of the body and at its tip becoming a free flagellum. The proportions of the total flagellar length represented by the free, attached, and flagellar pocket portions vary with the morphological type and with the species.

The whole length of the axonemal portion of the flagellum consists of the conventional array of a central pair of MTs and a peripheral set of nine doublets. Little attention has been paid to the detailed structure of the axoneme, but scrutiny of published micrographs of adequate resolution containing cross-sections of flagella indicates that it conforms in the details of its structure to the organization typical of cilia and eucaryotic flagella, with the exception that each B-tubule of the peripheral doublets bears a longitudinal partition (17, 22, 212). In addition to these axonemal structures, a trypanosomatid flagellum typically contains a paraxial rod (intraflagellar structure of Brun), which begins somewhere along the portion of the flagellum contained within the flagellar pocket and extends to the tip of the flagellum. In electron micrographs this paraxial rod has a regular, lattice-like organization, but its composition, three-dimensional structure, and function are not known. It is absent from the flagella of *C. oncopelti*, *B. culicis*, and probably *Crithidia deanei*. Comparisons of flagellar activity in these three species

and in other species might result in an understanding of the function of the paraxial rod; isolation and characterization of the components absent from the flagella of these three species but present in other species will certainly reveal its biochemical nature.

The major specializations of the trypanosomatid flagellum occur in its membrane, which lacks mastigonemes and is continuous at its base with the membrane of the flagellar pocket and through it with the membrane of the body surface. The flagellar membrane differs from these other membranes in the density of intramembranous particles on its protoplasmic and external faces (59, 66). One of the conspicuous and frustrating properties of the lower trypanosomatids is their capacity to attach firmly to each other or to debris in the culture medium to form large masses (rosettes), to the walls of culture vessels, pipettes, and centrifuge tubes, and to the surfaces of fibers of membrane filters. In their natural hosts, a large proportion of the cells of all species are attached to the epithelial cells or secreted linings of the host organs in which they occur. Brooker (16) has shown that all of these attachments are characterized by circumscribed thickenings of the inner leaflet of the flagellar membrane, beneath which lies a matlike array of filaments; no extracellular material could be seen above these specializations, which Brooker termed hemidesmosomes. Exposure to distilled water results in detachment, which begins by invaginations of these attachment sites, followed by the formation of vesicles in a process reminiscent of the processes described for the hemidesmosomes of tissue cells (252). Laugé and Nishioka (158) describe identical attachment sites in *L. oncopelti*.

Where the flagellum emerges from the flagellar pocket, its membrane bulges toward an extension of the rim of the pocket so that the two membranes are closely apposed. Desmosomelike specializations occur in groups or rows on the side opposite the paraxial rod. The detailed structures of the specialization differ in different species (14, 21, 166), but their general structure consists of dense plaques of different sizes beneath both membranes, sometimes with a thickening of the inner leaflet of the flagellar membrane but never of the pocket membrane, and filamentous processes extending across the narrow intermembrane space. Freeze-fracture preparations show groups or files of intramembranous particles on the protoplasmic face of the pocket membrane (21) or flagellar membrane (166); no such differentiation occurs in the apposed membrane. This region of specialized attachment sites appears to be the point of breakage when detergent-weakened flagella are removed by mechanical forces (213). Although several descriptions of attachment sites between flagellar membrane and body surface along the undulating membrane have been published (21), the brief account of Paulin (209) of two or three rows of "maculae adherentes" and corresponding small clusters of intramembranous particles along the apposed flagellar and plasma membranes of *B. culicis* is the only indication that the short undulating membrane of these epimastigotes is identical in construction to the undulating membranes of trypanosomes.

Proximally, just anterior to the point where the flagellar and pocket membranes become continuous, the central pair of MTs ends at a transverse partition and axial granule. Just proximal to this partition in *L. collosoma* and probably in other species, champagne glass structures (166) connect each doublet to the flagellar membrane, and filamentous projections extend between the pocket and flagellar membranes; typical "ciliary necklaces" have not been demonstrated (59, 166).

The peripheral doublets continue proximally to a second, incomplete transverse plate, where a third MT is added to each doublet to form the typical kinetosome. The proximal end of one triplet is sometimes described or shown as bearing a rootlet or filamentous process, which may extend to the nearby kinetoplast. It is not clear whether absence of this structure from most accounts and figures reflects its occurrence in only a few species or a failure to observe it because of unfavorable sections. It has been proposed as the morphological basis for the close association of kinetosome and kinetoplast maintained during morphogenetic changes and persisting in cell-free homogenates in the presence of Ca^{2+}.

The conventional construction of these flagella is paralleled by their conventional biochemistry and molecular biology in most respects. Pereira et al. (213) showed that isolated flagella from *H. samuelpessoai*, *C. fasciculata*, and *L. tarentolae* gave patterns qualitatively identical to those of flagella from other sources; tubulins were the most conspicuous components. Immunization of mice with preparations of flagella from *H. samuelpessoai* and *C. fasciculata* gave some protection against infection with *T. cruzi*. Glycerin-treated preparations of whole cells in which flagellar activity can be reactivated by adding an adenosine triphosphatase-containing salt solution are so easily prepared that such preparations can be used in classroom exercises (5).

Although simple microscopic examination of swimming and attached cells of several genera show that these flagella are capable of a consid-

erable variety of movements, systematic studies of their hydromechanical properties and control have been carried out only with *C. oncopelti* (39, 88, 89). The behavior of these flagella is more in accord with the sliding MT hypothesis than with the local contraction hypothesis. The bending activity produces a sinusoidal wave which is not quite planar, so that the cell rotates at a low frequency; the wave normally propagates from tip to base, but when the flagellum touches an obstruction anywhere along its length, the direction of propagation may be reversed. Micromanipulation and laser experiments show that the beat can be initiated at points other than the tip or base, and beating and reversal of direction of propagation in isolated fragments show that these properties are inherent in the flagellum itself and do not require any structures restricted to the portions of the flagellum proximal to the rim of the pocket. In both glycerol-extracted cells and live cells exposed to calcium ionophores, tip-to-base propagation occurs only at low Ca^{2+} concentrations; high concentrations reverse the direction of propagation. Since reversal of direction can also occur in normal cells in a medium containing Ca^{2+} chelators, Holwill and McGregor (128, 129) proposed that the direction of propagation is determined by internal Ca^{2+} concentration; this is normally low, but upon stimulation of any part of the cell surface, Ca^{2+} is released from internal sites of sequestration, modifies the interactions among axonemal MTs, and produces reversal of direction of propagation. Resequestration of Ca^{2+} over a period of several beats results in the return of the normal direction of propagation of the flagellar wave. The sites of sequestration may be in the membrane, but there are no reports of studies of the Ca^{2+}-binding properties of the membrane or of the possible occurrence of Ca^{2+} modular proteins, such as those which are found in other Ca^{2+}-regulated systems. The form of the wave requires that chemical energy be available at all points along the flagellum (126), but how the energy (as adenosine triphosphate) is continuously supplied is unknown. Calculations show that diffusion from a site of synthesis at the base is compatible with the energetics of beating; this conclusion is supported by the continued beating of a flagellum attached to an empty, broken cell so long as it is supplied exogenously with adenosine triphosphate. Goldstein et al. (89) point out that clear differences between the properties of the flagella of *C. oncopelti* and those of echinoderm sperm raise doubts about the applicability to all flagellar systems of conclusions based on investigations of flagella of *C. oncopelti*; the absence of the characteristic paraxial rod from these flagella similarly raises questions about applicability to trypanosomatid flagella generally.

Observations of living epimastigotes of *B. culicis* and of a variety of trypomastigotes show that bending movements occur in the portion of the body bearing the undulating membrane. Although these movements are of much smaller amplitude and longer wavelength than those occurring simultaneously along both the free and attached portions of the flagella, it seems reasonable to attribute them to flagellar activity rather than to the pellicular MTs. The latter interpretation would require attributing to a portion of the lengths of these MTs properties lacking in the remaining lengths. What advantage this effect of the presence of an undulating membrane confers on these body forms apparently has never been seriously considered.

Endosymbiotes

Three species (*C. oncopelti*, *C. deanei*, and *B. culicis*) are known to harbor cytoplasmic structures with the general properties of procaryotes except for the absence of a cell wall (28, 84, 98, 147, 191). These structures, originally termed bipolar bodies or diplosomes, are now recognized as intracellular symbiotes considerably integrated into the physiology of the host cell. They retain their own DNA of characteristic buoyant density and a genomic complexity typical of bacteria (273); their ribosomal RNA comigrates with *Escherichia coli* ribosomal RNA in acrylamide gels (250), and they have a chloramphenicol-sensitive protein-synthesizing system (147) and characteristic antigens (137). They are surrounded by a double membrane. The inner membrane is interpreted as the plasma membrane of the symbiote, and the outer membrane is interpreted as part of the membrane system of the host cell. Their division is coordinated with that of the host cell, so that they are distributed to daughter cells with as much precision as its own organelles. Their anabolic activities supply their hosts with several essential nutrients which other species of trypanosomatids must have supplied in the culture medium.

Symbiote-free strains of all three species have been produced by growth of the hosts in the presence of chloramphenicol (28, 190). A comparison of the infected and symbiote-free strains shows that the endosymbiotes contain uroporphyrinogen I synthetase (31) or ornithine carbamylase (24, 80) or both, enzymes which the host cells themselves can synthesize at very low rates or not at all, with the result that unlike other trypanosomatids, the host cells need no exogenous supply of heme and can use exogenous ornithine in place of arginine. Since both of the *Crithidia* species have quite simple nutri-

tional requirements when infected, whereas the cured strains have the complex requirements typical of the lower trypanosomatids, the endosymbiotes must also supply either the finished forms of amino acids and vitamins or usable intermediates. The contribution of the endosymbiote to *B. culicis* must be quite different; infected cells still require the hemin and amino acids typical of the lower trypanosomatids, but cured cells require an unidentified factor supplied by fetal calf serum or by extracts of whole blood (29, 30, 32). Although differences in cell surface saccharides occur in the infected and cured strains of *B. culicis* (64), Chang found no differences in agglutinability of infected and cured strains by homologous and heterologous antisera prepared against whole cells.

Although cultivation of the cured strains of these flagellates is no more difficult than cultivation of the more fastidious species of trypanosomatids which lack endosymbiotes, cultivation of the endosymbiotes themselves (freed by gentle rupture of the host cells) has not been possible. This failure may as likely be the result of the osmotic fragility of the naked endosymbiotes as of any unusual biochemical fastidiousness. Until such cultivation is possible, reinfection experiments are not feasible. Such experiments would be particularly interesting to test host specificity and perhaps determine how the mechanical problems of entrance of such relatively large objects (ca. 0.5 by 2 μm) into the host are overcome. Obviously, these systems offer choice experimental material for analysis of the development of interdependence in symbiosis and food for thought for all biologists, whether or not they espouse the hypothesis of the procaryotic symbiotic origins of mitochondria and chloroplasts.

ADDENDUM

It now seems likely that the microbodies of trypanosomatids are not in any way comparable with the microbodies of other cells but are the sites of glycolysis. F. R. Opperdoes (personal communication) finds in *C. luciliae* that, except for enolase, the enzymes of glycolysis are located in particles banding at a density of 1.23 g/cm^3 in sucrose gradients and containing in addition the marker enzyme NAD$^+$-linked glycerol-3-phosphate dehydrogenase, properties which are characteristic of the glycosomes of *T. brucei*, which by electron microscopy are microbodies in the conventional sense (FEBS Lett. **80**:360–364). He concludes that the portion of glycolysis from glucose to 3-phosphoglycerate proceeds in these glycosomes, not in the cytosol.

D. L. Fouts and D. R. Wolstenholme (Nucleic Acids Res. **6**:3785–3804) report that both whole cell and mitochondrial RNAs from *Crithidia acanthocephali* contain components which are complementary to the heavy strands but not to the light strands of kDNA minicircles isolated from this species; S1 nuclease digestions and electron microscopy of the RNA-DNA hybrids show that hybridization involves a single region of each kDNA heavy strand equal to about 10% of the contour length of the minicircle. They therefore suggest that in this species, in contrast to *C. luciliae* and *L. tarentolae*, the kDNA minicircles are transcribed.

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